The role of anchorage in cell cycle control

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Declaration

I, Catherine Anne Cremona, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed……Date……..

Abstract

Mammalian cells generally require both mitogens and anchorage signals in order to proliferate. Failure to receive these signals results in either cell-cycle arrest or cell death, known as anoikis, due to activation of anchorage-dependent checkpoint mechanisms. Transformed cells alleviate these checkpoints, via activation of oncogenes and/or inactivation of tumour suppressors. In contrast, detachment of normal cells halts cell-cycle progression in G1, because of insufficient cyclin D1 induction to overcome the Rb/E2F checkpoint, accumulation of cyclin-dependent kinase inhibitors, and lack of cyclin A production. Because of the complexity of anchorage-dependent mechanisms involved, several changes are required for cells to become anchorage independent: impairment of both Rb and p53 pathways, plus activation of Ras. These specific defects are found in many human cancers, and anchorage independence correlates strongly with tumourigenic potential. Here I have used cells expressing SV40 LT, with and without oncogenic Ras, to model the changes leading to anchorage independent proliferation. Importantly, cells expressing SV40 LT alone retain their anchorage dependence, despite Rb and p53 inactivation. However, the mechanism responsible for this cell-cycle arrest in suspension is not known. Using immunoprecipitation and kinase assays, I demonstrated that the cyclin-dependent kinase inhibitor p27 curbs cell-cycle progression in these cells. FACS analysis showed that surprisingly, these cells do not undergo a robust checkpoint arrest, but instead stall throughout the cell cycle, showing abnormal DNA replication. Further investigation by metaphase spread showed the accumulation of aneuploid nuclei, indicating the development of genomic instability. This resulted in a higher rate of transformation among cells cultured without anchorage for a limited time. This work demonstrates that anchorage signals enable proper activation as well as assembly of cyclin-dependent kinase complexes, and that adhesion is particularly important for maintaining orderly cell-cycle progression and preventing genomic instability in checkpoint-deficient cells.

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Table of contents

Table of Figures

Abbreviations used in this thesis

APS	Ammonium persulphate
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia related
BSA	Bovine serum albumin
CAK	CDK-activating kinase
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
DMEM	Dulbecco's modified Eagle medium
DMP	Dimethyl pimelimidate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCS	Foetal calf serum
GGF	Glial growth factor
HRP	Horseradish peroxidase
LB	Luria broth
LT	Large T antigen
MAPK	Mitogen-activated protein kinase
MCM	Minichromosome maintenance
MEF	Mouse embryo fibroblast
MEK	MAPK/ERK kinase
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide thiazole Blue
NIH 3T3	National Institutes of Health 3-day transfer of 3 $x10^5$ cells
NRK	Normal rat kidney fibroblasts
NS	Normal Schwann cells
NSLT	Normal Schwann cells expressing LT

NSLTRas	Normal Schwann cells expressing LT and oncogenic Ras
PBS	Phosphate-buffered saline
PDCA	Poorly-differentiated carcinoma
PI	Propidium iodide
PI3K	Phosphoinositide-3 kinase
PLL	Poly-L-Lysine
PMSF	Phenylmethylsulphonyl fluoride
RNA	Ribonucleic acid
RT	Room temperature
s.d.	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
s.e.m.	Standard error of the mean
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOC	Super optimal broth with catabolite repression
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.05% Tween 20
TEMED	Tetramethylethylene diamine
WT	Wild type
w/v	Weight to volume

Chapter One – Introduction

1.1 The cell cycle

Cell division is a fundamental property of living things, enabling the reproduction of unicellular organisms such as yeasts, bacteria and amoebae, and the development and maintenance of multicellular organisms from humans to jellyfish. The cell cycle is the process by which one cell gives rise to two cells, and the main aim of this complex sequence of events is to accurately reproduce and equally segregate genetic material between the two daughter cells. This allows coordinated expression from an identical genome throughout the organism, and enables inheritance of genetic traits by the cellular offspring. In addition, regulatory mechanisms ensure that each new cell contains the means to support itself via energy production, and the structural components to maintain itself as an entity. Further mechanisms determine when and how a cell enters and leaves the dividing state. For over 50 years, cell division has been investigated at the molecular level, and new complexities are still being revealed.

Mutations and defects at all levels of cell cycle regulation may lead to uncontrolled cell proliferation and cancer. Given the many trillions of cell divisions that take place over the lifetime of a human, these regulatory mechanisms are remarkably effective at guarding against tumourigenesis. Nevertheless, cancer was responsible for 13% of all deaths worldwide in 2007, according to the World Health Organisation, and thus remains the subject of intense research. Although there are differences in cell cycle regulation between different species and cell types, the broad similarities in the process and the remarkable conservation of the underlying genes and proteins involved has meant that important breakthroughs in cancer research have been made using model organisms as diverse as yeast, flies, frogs and mice. This chapter will introduce the key aspects of cell cycle regulation relevant to the research in mammalian cells presented in this thesis.

1.1.1 Phases of the cell cycle

To describe the eukaryotic cell cycle, it has historically been divided into phases, at first on the basis of simple microscopic observation, and then further subdivided, as more biochemical processes which occur at specific times in the cell cycle have been described. Initially, proliferating cells were observed in either 'mitosis', partitioning their chromosomes equally into daughter nuclei, or 'interphase', the intervening period between cell divisions. 'M' phase refers to both segregation of the genome at mitosis, and to the physical separation of cytoplasm to contain the two new nuclei in separate daughter cells, known as cytokinesis. Within interphase is the DNA synthesis or 'S' phase, where the genome is replicated. The exact replication of the genome to produce two identical copies, and the inheritance of precisely one copy of the genetic material by the two daughter cells, are the fundamental objectives of the cell division cycle. The simplest cell cycles therefore consist of alternating S and M phases, such as the early embryonic cell divisions of the frog *Xenopus laevis*, and the syncytial nuclear divisions that take place in the embryo of the fly *Drosophila melanogaster* (where cytokinesis is also absent). However, these are special cases, when rapid divisions take place without growth in order to create a multicellular embryo from the large fertilised egg. In most cases, S and M phases are separated from each other by two 'gap' phases designated G1 (post-mitosis) and G2 (pre-mitosis). This allows time for cell growth, to maintain the average size of cells in a homeostatic population. During this time, extracellular signals from growth factors, mitogens, surrounding cells and extracellular matrix proteins are also integrated to control cell cycle progression and timing. Clear separation of S and M phases means that the key processes of genomic duplication and division are insulated from each other, so that cell division cannot occur before DNA replication is complete, and vice versa. This ensures faithful inheritance of the complete genome by each cell and thus assures the future competence of the organism.

In special cases, cell division occurs without DNA replication, as in meiosis to produce the haploid gametes; or DNA replication occurs without cell division, as in the endoreduplication cycles of the placental trophoblast giant

cells in mice. However, these examples require specific controls, which override normal cell cycle progression.

In addition to the equal segregation of genetic material, organelles such as mitochondria and Golgi must be distributed between the two daughter cells. This is not a passive process, and is achieved by association of the fragmented organelles with components of the cytoskeleton (Shima et al. 1998; Boldogh et al. 2001). Other key proteins are also actively distributed between daughter cells, sometimes accumulating in a polarised manner in the mother cell and segregating predominantly to one of the two daughter cells. This is known as asymmetric division, which is especially common during development as a mechanism of producing cells with different fates, and also occurs in the production of differentiated cell types from stem cells, where one daughter is retained as a stem cell.

In a continually proliferating population, the four cell cycle phases repeat in a loop (Figure 1- 1), so that newly produced cells from M phase return to G1 and continue to S phase, then G2, and so on. This results in a doubling of the cell population with each completed cell cycle, leading to exponential population growth under ideal conditions. Conversely, in the absence of mitogenic factors, or the presence of inhibitory signals, newborn cells stop cell cycle progression, known as 'arrest', and may exit from the cell cycle altogether to a state known as 'quiescence' or G_0. Quiescent cells are still receptive to external signals even though they are not actively dividing, and the cell cycle arrest is reversible following re-stimulation with mitogens, allowing cells to return to the cell cycle. Importantly, early cell culture experiments using serum as a source of mitogens found that although serum withdrawal in early G1 phase led to quiescence, removal of mitogen signals at later phases did not immediately arrest the cells, and they were able to complete one cell cycle and return to G1 before arresting (Temin 1971; Zetterberg and Larsson 1985). This transition from a mitogen-dependent to a mitogen-independent state should ensure that S and M phases are always completed once started, regardless of the persistence of external signals, so that cells are not left with partially replicated or separated DNA. The point of commitment to cell division was classically known as the 'restriction point', from which cells enter quiescence and where

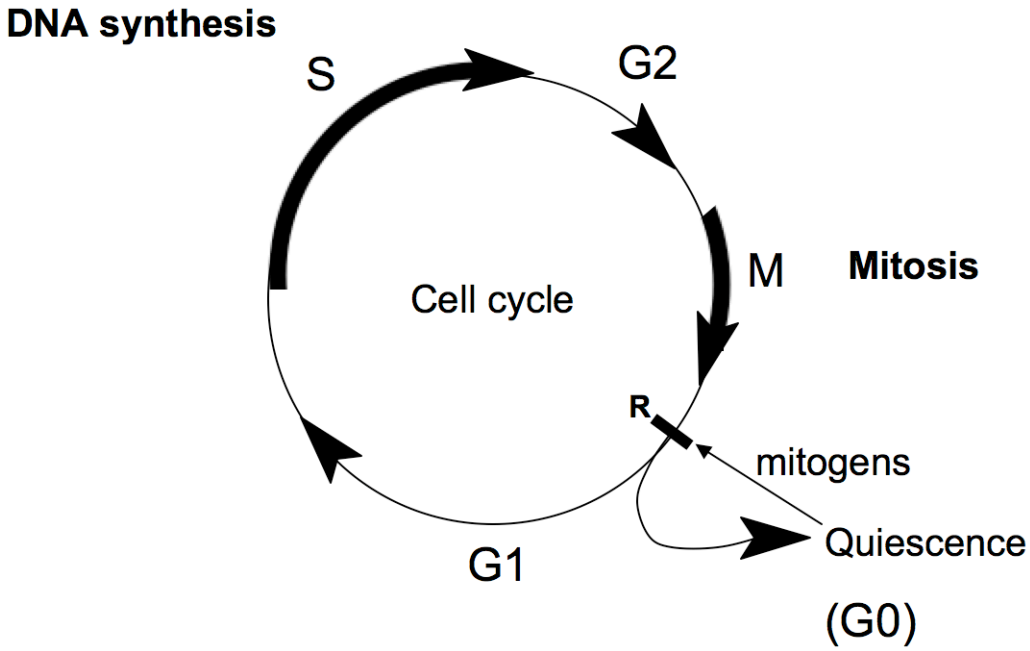


Figure 1- 1: Phases of the cell cycle.

Diagram shows the relative positions of the mitotic (M), DNA synthesis (S) and gap (G1, G2) phases in the mammalian cell cycle. R indicates Pardee's restriction point, where cells may exit the cycle to the quiescent (G0) state, and where cell cycle progression becomes mitogen-independent (see text). Diagram adapted from review (Baserga 1965).

they re-enter the cell cycle when stimulated with mitogens (Pardee 1974). More permanent exit from the cell cycle occurs when cells cease to proliferate in response to mitogens: for example in cellular senescence following environmental stress (Blagosklonny 2003), or physiologically, during cell differentiation (Walsh and Perlman 1997).

The process of mitosis can itself be subdivided into stages according to observable features visible under the microscope (Figure 1- 2). These include:

(1) Prophase, in which chromosomes condense. Sister chromatids of replicated DNA produced in S phase remain held together by cohesin rings. Duplicated centrosomes separate and move to opposite poles.

(2) Prometaphase, where the nuclear envelope breaks down and microtubules form a 'spindle' from opposite cell poles around the condensed chromosomes. Microtubules attach to the central kinetochore of each chromosome.

(3) Metaphase, where chromosomes stably attached to both spindle poles align at the equator of the cell. Kinetochores are under tension, produced by motor forces on opposing spindle microtubules pulling towards the poles.

(4) Anaphase, the separation of the chromatid pairs to opposite sides of the cell. When cohesin rings are cleaved, tension is released, the opposing forces acting on the kinetochores pull the sister chromatids apart, and they move towards the spindle poles.

(5) Telophase, the reformation of nuclear envelope around each new set of chromosomes, and decondensation of the chromatin.

These stages are all continuous, not discrete steps, with the important exception of the metaphase to anaphase transition, which occurs suddenly and only upon successful bipolar attachment of every chromosome to the mitotic spindle. This is a key control point in the cell cycle, and will be discussed in more detail later.

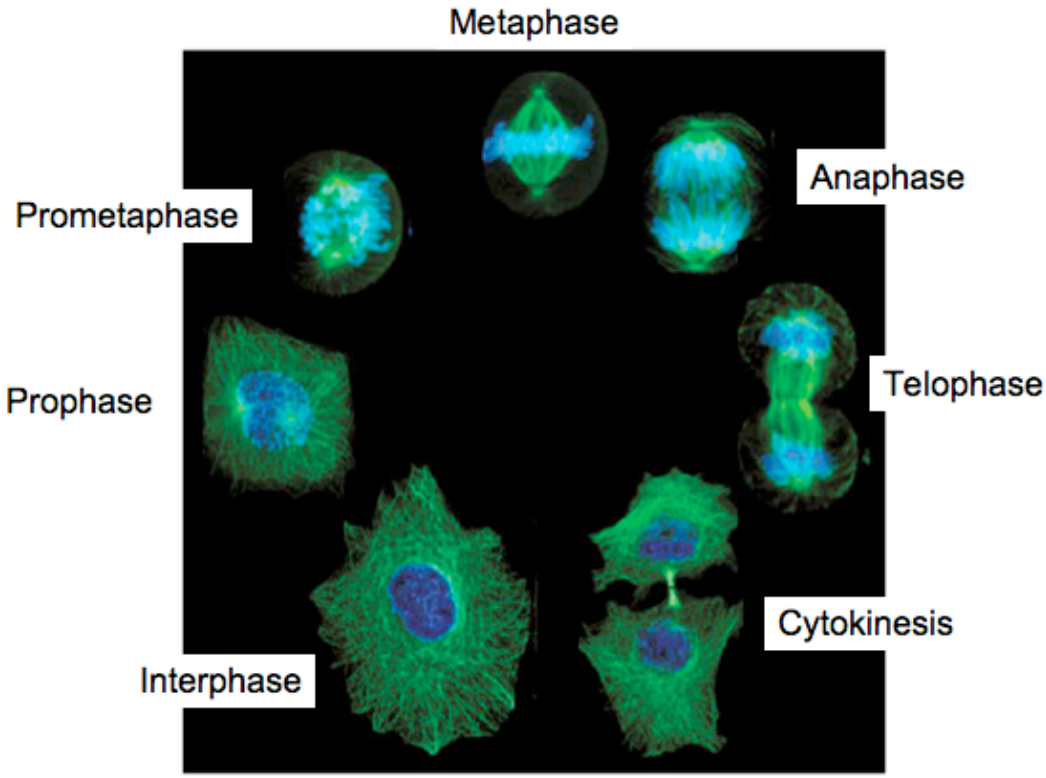


Figure 1- 2: Stages of the cell cycle as seen by fluorescence microscopy.

Stages of mitosis (prophase, prometaphase, metaphase, anaphase, and telophase) are shown, along with cytokinesis and interphase (see text). Chromatin is indicated in blue and microtubules in green. Illustration adapted from Wan laboratory, University of Pittsburgh Cancer Institute.

The progression from G1 to S phase to G2 to M phase and through mitosis and cytokinesis must be strictly controlled in sequence to ensure that each cell inherits a complete, intact genome. Two key processes drive cell cycle progression forward: phosphorylation of target proteins by cyclin-dependent kinase (CDK) complexes; and protein ubiquitination, notably by the anaphase-promoting complex/cyclosome (APC) and the Skp1/cullin/F-box protein complex (SCF), which tag proteins for degradation by the proteasome. The phosphorylation of proteins is readily reversible, and determines their activity and/or structural conformation, often restricting activity to a particular period of time. Proteolytic degradation, in contrast, is irreversible, and allows rapid removal of proteins once they are no longer needed, facilitating the transition forward to the next stage of the cell cycle. Both mechanisms are partly self-regulating, in that CDK complexes are themselves controlled by CDK-mediated phosphorylation (Fisher and Morgan 1994; Welcker et al. 2003), and both APC and SCF components are themselves targets for APC-mediated ubiquitination (Shirayama et al. 1998; Bashir et al. 2004; Wei et al. 2004). Additionally, the CDK phosphorylation and APC ubiquitination mechanisms are mutually regulating (Zachariae et al. 1998). Robust, irreversible switching from one cell cycle stage to the next is achieved by this complex network of protein signalling, including both positive and negative feedback loops (Novak et al. 2007). The following sections describe the individual proteins involved in more detail.

1.1.2 Cyclin-CDK complexes control cell cycle progression

1.1.2.1 Discovery of cyclin-CDK complexes

The key drivers of cell cycle progression are the cyclin-dependent kinases or CDKs, which are activated at specific times, primarily by the binding of protein partners known as cyclins. These were named due to their regular appearance and disappearance in sea urchin eggs over time, with a periodicity that corresponded to the timing of the cell cycle (Evans et al. 1983). Historically, the existence of the canonical cyclin-CDK complex as a controller of cell division was elucidated by a combination of data from yeast (both *Saccharomyces cerevisiae* and *Saccharomyces pombe*) and frog oocytes (*Rana*

pipiens and *Xenopus laevis*), as well as clam and sea urchin eggs. In yeast, screens of temperature-sensitive mutant strains identified several 'cell division control' (cdc) mutants which failed to divide at the restrictive temperature: in particular, the cdc28 mutant of *S. cerevisiae* (Hartwell et al. 1974) and the cdc2 mutant of *S. pombe* (Nurse and Thuriaux 1980). cdc28 and cdc2 genes were later found to be homologous (Beach et al. 1982), and both encoded a protein with kinase activity (Reed et al. 1985; Simanis and Nurse 1986). Meanwhile, an unknown activity present in mature oocyte cytoplasm was found to promote meiosis of immature frog oocytes and named 'maturation-promoting factor', or MPF (Masui and Markert 1971; Smith and Ecker 1971). Cytoplasm from an M-phase cell, but not an interphase cell, induced M-phase entry when injected into an immature oocyte. A similar factor causing frog oocyte maturation was found to be present in mitotic human cells (Sunkara et al. 1979). Following the initial discovery of cyclins A and B in sea urchin oocytes (Evans et al. 1983), cyclin A was cloned from clam embryos, and the mRNA was found to trigger M phase entry in Xenopus oocytes (Swenson et al. 1986). MPF was purified from *Xenopus* oocytes and found to be composed of two subunits of approximately 34 and 45kDa (Lohka et al. 1988), which were later discovered to be homologous to cdc2 kinase and cyclin B respectively, by binding to antibodies against those proteins (Gautier et al. 1988; Gautier et al. 1990). The clam homologue of cdc2 was found to bind both cyclins A and B, and both complexes had cell cycle-dependent kinase activity (Draetta et al. 1989). These discoveries established the concept of the cyclin-dependent kinase complex in regulating cell division. Several cyclin and CDK proteins homologous to those already found in clam, sea urchin, *Xenopus* and yeast were rapidly found in several species, including humans. The level of homology between species is such that human CDK1 can rescue a yeast cdc2 mutant, as seen below. Proteins are listed according to the mammalian nomenclature, for clarity.

Protein	Organism	Discovery	References
Cyclin B	Yeast (*S. pombe*)	As 'cdc13', mutant genetically associated with cdc2; similar to clam cyclin; involved in mitosis	(Hagan et al. 1988)
Cyclin B1 & B2	*Xenopus*	*Xenopus* oocyte cDNA library probed with sea urchin cyclin B cDNA	(Minshull et al. 1989)
Cyclin B	Human	HeLa cell cDNA library probed with consensus sequence from clam, sea urchin, frog, fly and yeast cyclins; association with human cdc2 homologue (CDK1)	(Pines and Hunter 1989)
Cyclin A	Fly (*Drosophila melanogaster*)	cDNA library probed by consensus sequence of clam and sea urchin cyclin; cell cycle-dependent degradation	(Lehner and O'Farrell 1989)
Cyclin A	Human	cDNA of gene locus disrupted by HBV integration in liver cancer; homology to clam and fly cyclin A	(Wang et al. 1990)
Cyclin D	Yeast (*S. cerevisiae*)	As 'CLN1 and CLN2', rescued cdc28 mutation; homology with cyclins; act in G1 phase	(Hadwiger et al. 1989)
Cyclin D1	Human	Human cDNA complementation of CLN-deficient yeast; relation to A and B-type cyclins	(Lew et al. 1991; Xiong et al. 1991)
Cyclin E	Human	Rescue of CLN-deficient yeast; homology to A and B cyclins; genetic interaction with human CDK2 in yeast	(Koff et al. 1991; Lew et al. 1991)

Protein	Organism	Discovery	References
CDK1	Human	Human cDNA library complementation with cdc2 mutant yeast; binds cdc2 antibody	(Draetta et al. 1987; Lee and Nurse 1987)
CDK2	*Xenopus*	As 'Eg1' in cDNA screen for proteins synthesised in oocytes but not embryos; homology to cdc2	(Paris et al. 1991)
CDK2	Human	Complementation of cdc28 mutant; homology to cdc2 and Eg1	(Elledge and Spottswood 1991; Ninomiya-Tsuji et al. 1991)
CDK2	Human	Clone of human cdc2-related gene; homology with Eg1; binds cyclin A	(Tsai et al. 1991)
CDK4	Human	cDNA screen for protein-serine kinases in HeLa cells; homology to cdc2 and cdc28	(Hanks 1987)
CDK4	Mouse	As 'p34PSK-J3', catalytic subunit of D-type cyclins	(Matsushime et al. 1992)
CDK6	Human	Protein kinase homologous to cdc2; binds D cyclins in human cells	(Meyerson et al. 1992; Meyerson and Harlow 1994)

In all, 20 'CDK' proteins and 29 'cyclin' genes were identified from human sequence data as having sequences similar to known cyclins and CDKs, although just a few of these have proven activity directly linked to cell cycle progression: cyclins A, B, D, E and H, and CDKs 1, 2, 4, 6, and 7 (Malumbres and Barbacid 2005).

Cyclins were found to bind their CDK partners via a conserved, 150 amino-acid 'cyclin box' sequence identified by point mutation of amino acid

residues in cyclin A (Kobayashi et al. 1992). Although the CDKs may bind promiscuously to different cyclins under certain conditions, they characteristically bind particular cyclin partners: CDK1 with either cyclin A or cyclin B, CDK2 with either cyclin E or cyclin A, and both CDK4 and CDK6 with the D-type cyclins (Draetta et al. 1989; Pines and Hunter 1989; Tsai et al. 1991; Koff et al. 1992; Matsushime et al. 1992; Rosenblatt et al. 1992; Meyerson and Harlow 1994). In most cases, CDKs are inactive in the absence of cyclin binding, but binding to cyclin alone is not sufficient for full activation (Desai et al. 1992; Connell-Crowley et al. 1993). CDK activation is controlled by a combination of three mechanisms: cyclin binding, phosphorylation on specific residues, and binding of CDK inhibitors (CDKIs).

1.1.2.2 Control of CDK activity by cyclin binding and proteolysis

Cyclin binding is considered to be the primary mode of CDK regulation, since the conformational change in the CDK it induces is necessary for access to the regulatory phosphorylation residues (Jeffrey et al. 1995). The presence of the cyclin subunit therefore defines the period during which the CDK complex is competent for activation, and regulation of cyclin levels allows cyclical regulation of CDK activity. Cyclin levels are mostly regulated in a cell cycle-dependent manner, by both transcriptional and degradative mechanisms (Figure 1- 3). Cyclin degradation especially is important for transitions between phases of the cell cycle, in particular the exit from mitosis, as described later in this section.

Cyclin E accumulates from mid-G1 to reach maximum levels at the G1-S phase transition, and then declines on S phase entry, at both the mRNA and protein level (Koff et al. 1992). Cyclin A and B mRNAs also oscillate mirroring the protein levels, with cyclin A levels peaking at the beginning of mitosis, whereas cyclin B levels peak slightly later, at the metaphase-anaphase transition (Pines and Hunter 1990; Whitfield et al. 1990). Accumulation of cyclin A starts before that of B, at the start of DNA replication, while cyclin B levels begin to rise in mid-S phase. Both have similar CCAAT sequences in their promoter and are regulated by the CCAAT-binding transcription factors CBP (Cyclin A), which is dependent on cell adhesion, and NFY (Cyclins A and

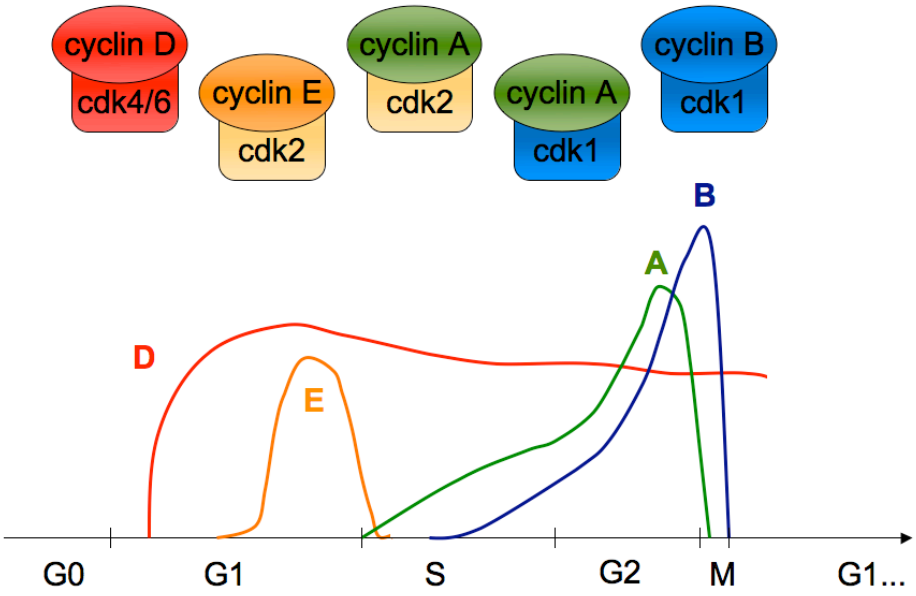


Figure 1- 3: Expression of the cyclins.

Cartoon indicates order of activation of the cyclin-CDK pairs (L-R) during one cell cycle. (NB. Level and duration of activation is not shown.)

Graph shows approximate expression patterns of the cyclins throughout a model cell cycle, starting with return from quiescence (G0). G1, S, G2, M denote gap 1, DNA synthesis, gap 2 and mitotic phases respectively. For clarity, different cyclin isoforms (D1, D2 etc.) are not included. Note that the protein level does not necessarily correspond to the level of associated kinase activity: for example, cyclin B-CDK1 is only activated at the beginning of mitosis by cdc25 dephosphorylation, despite the protein complex being present in G2 phase. Adapted from Sherr 1996.

B). Interestingly, transactivation by NFY increases during S phase as it is activated by cyclin E/A-CDK2, which may explain the later rise in cyclin B transcription (Henglein et al. 1994; Katula et al. 1997; Krämer et al. 1997; Bolognese et al. 1999; Erlandsson et al. 2000). Unusually, cyclin B transcription continues during mitosis, with the NFY transcription factor remaining bound to the active promoter in HeLa cells (Sciortino et al. 2001). This is very rare, since almost all RNA PolII-transcribed genes are inactive in M phase. Cyclin E and A genes also have promoter binding sites for E2F-family transcription factors, which are themselves repressed by the key Rb family of tumour suppressor proteins. Overexpression of cyclin E overcomes Rb-mediated repression of the cyclin A promoter, and ectopic cyclin A expression can reverse Rb-mediated G1 arrest, evidence that both cyclins are downstream of Rb control (Ohtani et al. 1995; Schulze et al. 1995; Knudsen et al. 1999). Control of the cell cycle by Rb and E2F will be described more fully in the next section. A further cell cycle-dependent element/cell cycle genes homology region (CDE-CHR) is also found in the cyclin A promoter, in common with the CDK1 gene. CDF-1 binding to the CDE-CHR represses cyclin A transcription during G0 and G1 phases. (Zwicker et al. 1995; Liu et al. 1997b).

The only cyclin involved in the cell cycle whose expression is not dependent on cell-cycle phase is the cyclin D family. Induction of cyclin D mRNA is controlled coordinately by signalling from mitogens and the extracellular matrix, to regulate cell cycle entry from quiescence, acting as a so-called 'mitogen sensor' – mRNA levels are stable in continuously-cycling cells (Won et al. 1992; Müller et al. 1994; Böhmer et al. 1996; Aktas et al. 1997; Roovers et al. 1999; D'Amico et al. 2000; Zhao et al. 2001; Zhao et al. 2003). Cyclin D protein levels during the cell cycle are therefore controlled by ubiquitin-mediated degradation, and the protein turnover is fast to allow for a rapid response to mitogen withdrawal and/or loss of anchorage signals. Cyclin D1 has a very short half-life of less than 30 minutes when complexed with CDK and less than 15 minutes when free (Matsushime et al. 1991; Matsushime et al. 1992; Bates et al. 1994; Diehl et al. 1997). The phosphoinositide-3-kinase (PI3K) pathway antagonises glycogen synthase kinase (GSK)-3 beta phosphorylation of cyclin D1 on T286, which would mark it for proteolysis

(Diehl et al. 1998). This may explain why two 'pulses' of signalling are necessary and sufficient to commit quiescent cells to the cycle. The first stimulates induction of cyclin D mRNA via the MAPK pathway; the second prevents the GSK-3 beta tagging the cyclin D protein for destruction, via the PI3K pathway (Jones and Kazlauskas 2001; Blagosklonny and Pardee 2002).

GSK-3 beta is also implicated in targeting cyclin E for proteolysis, phosphorylating it on Thr380 (Welcker et al. 2003). It was initially thought that this was an autophosphorylation site for cyclin E-CDK2, based on mutation analysis of cyclin E stability and measurements of CDK2 activity (Won and Reed 1996), but the CDK2 target site is in fact Ser384, which binds the prolyl isomerase PIN1 and is recognised by the Cdc4 subunit of the SCF ubiquitin ligase complex, and may also affect cyclin E localisation. Thr62 phosphorylation by CDK2 has been shown to promote ubiquitin-mediated proteolysis of cyclin E by positively regulating Thr380 phosphorylation (Strohmaier et al. 2001; Ye et al. 2004; Yeh et al. 2006). When in complex with CDK2, Thr380-phosphorylated cyclin E is recognised by the Fbw7 subunit of the SCF ubiquitin ligase complex. CDK2 binding prevents recognition by Cullin-3, which targets free cyclin E for destruction. Any modification that disrupts cyclin E/CDK2 binding may therefore indirectly lead to cyclin E proteolysis (Clurman et al. 1996; Singer et al. 1999). More recent work has shown that the two cyclin E-recognising E3 ligases, Cdc4 and Fbw7, dimerise, and that this enhances turnover of cyclin E doubly phosphorylated at both Thr380 and Ser384 (Hao et al. 2007). Autophosphorylation of cyclin E by the cyclin E-CDK2 complex, leading to its degradation, is an important negative feedback loop limiting cyclin E expression. If cyclin E is overexpressed or unable to be degraded, it results in S phase abnormalities and increases the likelihood of genomic instability and malignancy (Strohmaier et al. 2001; Yeh et al. 2006).

Ubiquitin-mediated proteolysis of cyclins A and B is mediated by the APC complex, rather than SCF as for cyclins D and E. This cyclin removal is required for cell cycle progression: in the absence of cyclin B degradation, cells are unable to complete anaphase and cannot exit mitosis (Wolf et al. 2006). Even before the discovery of the APC, it was clear that destruction of cyclin B

was necessary for cell division, and that it occurred by a ubiquitin-dependent mechanism (Murray et al. 1989; Glotzer et al. 1991). Several genetic components required for cyclin B proteolysis were discovered in yeast (Irniger et al. 1995), and the multisubunit APC complex was purified from oocyte extracts of clam and *Xenopus* (King et al. 1995; Sudakin et al. 1995), pointing to an intricate system of protein regulation. Indeed, new APC functions are still being revealed (Diamond et al. 2009; Kim et al. 2009a). Cells arrested in prometaphase with the microtubule poison colchicine were able to degrade cyclin A, but not cyclin B (Hunt et al. 1992), so it was a surprise when they were both found to be targeted by the same complex. Cyclin A levels decline just after nuclear envelope breakdown, and the protein is degraded before anaphase, unlike cyclin B which is present at high levels until the metaphase-anaphase transition (den Elzen and Pines 2001; Geley et al. 2001). The mitotic cyclins both contain an N-terminal 'destruction box' which is recognised and bound by the APC (Glotzer et al. 1991; Luca et al. 1991; Sudakin et al. 1995; Meyn et al. 2002; Yamano et al. 2004).

Substrate recognition is mediated by the two APC activating subunits, Cdc20 and Cdh1, which have homologues in yeast, *Drosophila*, *Xenopus* and humans (Dawson et al. 1995; Visintin et al. 1997; Kitamura et al. 1998; Kramer et al. 1998; Lorca et al. 1998). Interestingly, APC targeting is controlled by CDK1 and Polo-like kinase phosphorylation on the complex itself (Sudakin et al. 1995; Kotani et al. 1998; Golan et al. 2002), in contrast to the SCF complex, where target recognition requires specific phosphorylations on the substrate cyclins (see above). Phosphorylation of APC^{Cdc20} promotes its activation, while phosphorylation of Cdh1 inhibits APC^{Cdh1} activity, and this difference helps to separate the proteolysis of different targets into two phases. The two APC activating subunits recognise different sets of target proteins and ensure that they are degraded sequentially: APC^{Cdc20} is active in metaphase, followed by APC^{Cdh1} activation in telophase (Kramer et al. 2000). APC^{Cdh1} activity continues past mitosis and into the following G1 phase, which prevents reaccumulation of cyclins A and B until after the G1-S phase transition (Amon et al. 1994; Irniger and Nasmyth 1997; Bastians et al. 1999). The APC complex

is finally inactivated in late G1 by ubiquitinating its own E2 ubiquitin ligase, UbcH10 (Rape and Kirschner 2004).

As well as targeting mitotic cyclins for proteolysis, a key role of the APC is in triggering the metaphase-to-anaphase transition, by causing the separation of chromatid pairs to opposite poles. It was first thought that destruction of cyclin B was sufficient for triggering anaphase, but these events were later found to be separable (Holloway et al. 1993; Surana et al. 1993). This observation was explained when APC^{Cdc20} was found to target securin at the metaphase-anaphase transition, while cyclin B was targeted by APC^{Cdh1} (Schwab et al. 1997; Visintin et al. 1997). The destruction of securin (Pds1/Cut2) proteins, as a result of APC activity, activates separase (Esp1/Cut1), a protease which cleaves the cohesin (Scc1) rings holding sister chromatids together and allows them to be 'pulled' apart by the spindle microtubules attached to their kinetochores (Ciosk et al. 1998; Uhlmann et al. 1999). This crucial event, and its initiation by the APC, is regulated by the spindle checkpoint, which will be discussed in section 1.1.3.3. Cyclin B and securin are thought to be the only essential targets of the APC, since yeast lacking the complex can divide if securin is deleted and a cyclin B inhibitor is overexpressed (Thornton and Toczyski 2003).

The degradation of cyclin B results in the rapid loss of CDK1 activity, which induces mitotic exit and cytokinesis. Experiments using inhibitors have dissected the relative importance of cyclin B removal and loss of CDK1 activity in the completion of mitosis (Potapova et al. 2006). Chemical inhibition of CDK1 activity in mitosis was sufficient to allow mitotic exit, even in the presence of cyclin B. If CDK1 inhibitors were washed out while cyclin B was still present, cells reverted to M phase, surprisingly even re-fusing after cytokinesis. Proteolytic removal of cyclin B therefore ensures irreversible CDK1 inhibition and forward progression to G1. The coordinated degradation of securin and cyclin B is also crucial for completion of mitosis: if CDK1 inhibition occurs without cohesin cleavage, cytokinesis will occur without chromatid segregation, trapping chromatin between the two cells and resulting in catastrophic damage to genetic material (Potapova et al. 2006).

1.1.2.3 Control of CDK activity by phosphorylation

In addition to the extensive mechanisms regulating the expression and degradation of the cyclins, CDK complexes (Figure 1- 3) are both positively and negatively regulated by phosphorylation. Since regulation by protein phosphorylation is virtually instantaneous, this establishes fast, 'switch-like' controls on CDK activity, and enables robust entry into distinct cell cycle phases.

CDK7 binds cyclin H to form the 'CDK-activating kinase' (CAK) holoenzyme (Fisher and Morgan 1994), which phosphorylates the other CDKs on a conserved threonine residue on the "T-loop" (Thr 160/161/172) to allow protein substrate access to the ATP binding site of the enzyme (Gu et al. 1992; Solomon et al. 1992; De Bondt et al. 1993; Kato et al. 1994). Negative regulation of CDKs by phosphorylation occurs notably at the Thr14/Tyr15 residue on CDK1 and CDK2 (Gu et al. 1992). The Tyr15 inhibitory phosphorylation is catalysed by Wee1 kinase, and removed by CDC25 phosphatase (Gautier et al. 1991; Parker and Piwnica-Worms 1992). A second, dual-specificity kinase, Myt1, can phosphorylate both Thr14 and Tyr15 inhibitory positions in *Xenopus* cdc2, and is conserved in humans (Mueller et al. 1995; Liu et al. 1997a). Activation of the cyclin B-CDK1 complex depends on the balance of kinase and phosphatase activity in the cell at the G2/M phase transition (Atherton-Fessler et al. 1994), and following cyclin B degradation at mitotic exit, CDK1 is phosphorylated to lock it into the inhibited state for G1 phase (Potapova et al. 2009). Whether other CDKs such as CDK4 are regulated by inhibitory phosphorylation is controversial. An inhibitory phosphorylation has been detected on CDK4 at a similar Tyr17 position, but it is induced under conditions of quiescence or arrest, rather than being necessary for normal cell cycle progression (Terada et al. 1995; Iavarone and Massagué 1997; Jinno et al. 1999). A CDC25 phosphatase was found to be responsible for the Tyr17 dephosphorylation, but the kinase is not Wee1 or Myt1 (Watanabe et al. 1995; Booher et al. 1997). The function of this phosphorylation seems to be less important than the Tyr15 phosphorylation on CDK1 and CDK2, since mutation of Tyr17 did not significantly affect cyclin D-CDK4 activity (Coleman et al. 1997).

1.1.2.4 Control of CDK activity by inhibitors

The third mechanism of regulating cyclin-dependent kinase activity is the binding of protein inhibitors to the CDK complexes. There are two main groups of CDK inhibitors (CDKI): the inhibitors of CDK4 (INK4) family and the CDK-interacting protein/ kinase inhibitory protein (Cip/Kip) family. The INK4 family inhibit CDK4 and CDK6 by direct binding of the CDK alone, which distorts both the ATP and cyclin-binding sites (Brotherton et al. 1998; Russo et al. 1998; McConnell et al. 1999; Jeffrey et al. 2000). The family includes p15(INK4b), p16(INK4a), p18(INK4c) and p19(INK4d). p16 was the first to be identified, as a CDK4 interactor in fibroblasts transformed with the viral oncoprotein SV40 LT, and by yeast 2-hybrid, which inhibited the kinase activity of the cyclin D-CDK4 complex (Serrano et al. 1993; Xiong et al. 1993b). p15 was then identified as a p16-related protein in cells arrested by the extracellular 'transforming growth factor' TGFß (Hannon and Beach 1994). p18 and p19 were identified by yeast 2-hybrid as CDK6 interactors (Guan et al. 1994; Guan et al. 1996). p16 expression was found to be repressed by the tumour suppressor protein Rb, which in turn is inhibited by the cyclin D-CDK4/6 complex, resulting in a negative feedback loop (Li et al. 1994). Since the activity of the cyclin D-CDK4/6 complex is crucial in stimulating cell cycle re-entry from quiescence, both Rb and p16 are key in controlling proliferation of quiescent cells, reflected by their frequent disruption in cancer (Ruas and Peters 1998; Sherr 2001a). Additionally, the INK4a locus encodes another tumour suppressor protein, ARF, which activates the transcription factor and most commonly mutated tumour suppressor protein, p53 (Levine et al. 1991). Losing expression from this locus, whether by deletion, mutation or silencing, will therefore remove two tumour suppressors at a stroke (Sherr 2001b). Further introduction to the role of tumour suppressors in cancer is given in section 1.3.

The Cip/Kip family of CDKIs consists of p21(Cip1), p27(Kip1) and p57(Kip2). p21 was discovered in 1993 as a CDK2 interactor by yeast 2-hybrid and by immunoprecipitation, as a CDK2 inhibitor, and as a p53 target (el-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993a). p27 was identified as a p21-related protein, an inhibitor for both cyclin A-CDK2 and

cyclin E-CDK2 that accumulates in G0- and G1-arrested cells and as a cyclin D-CDK4 interactor in proliferating cells (Hengst et al. 1994; Polyak et al. 1994a; Polyak et al. 1994b; Toyoshima and Hunter 1994). p57 was identified by homology to p21 and by yeast 2-hybrid as a cyclin D1 interactor, upregulated in terminally differentiated cells (Lee et al. 1995; Matsuoka et al. 1995). Later, the p57 gene was found to be imprinted in humans, with only the maternal allele expressed; and mutated in Beckwith-Wiedemann syndrome, which predisposes patients to cancer (Hatada et al. 1996). p57 is involved in regulating the endoreduplication cycles of DNA replication in trophoblast cells, by controlling CDK activity (Hattori et al. 2000).

All three family members preferentially bind cyclin-CDKs in complex. The N-terminal inhibitory domain of amino acids is conserved, and is both necessary and sufficient for CDK2 inhibition (Chen et al. 1995; Luo et al. 1995; Nakanishi et al. 1995; Chen et al. 1996a). When bound to cyclin-CDK, the inhibitory domain contacts both the cyclin box and the catalytic site of the kinase, effectively inhibiting both kinase activity and substrate recruitment by the cyclin (Russo et al. 1996). In stoichiometric experiments, one molecule of p21 was shown to be sufficient for complete inhibition of one cyclin A-CDK2 complex (Hengst et al. 1998) despite an earlier report suggesting a multiple p21:complex ratio might be needed for full CDK inhibition (Harper et al. 1995). Expression levels of both p21 and p27 peak in G1 phase, and they are both proteolytically regulated: p27 is targeted for ubiquitination by SCF^{Skp2} following phosphorylation by cyclin E-CDK2 on Thr187, and p21 similarly but by both phosphorylation-dependent and independent mechanisms (Sheaff et al. 1997; Vlach et al. 1997; Sheaff et al. 2000; Bornstein et al. 2003). p27 levels are therefore controlled by a positive feedback loop: in S phase, when cyclin-CDK2 complexes are active, Thr187 phosphorylation of p27 will tag it for proteolysis via SCF^{Skp2}, reducing the amount available to inhibit cyclin-CDK2 complexes and increasing CDK activity and p27 phosphorylation further (Malek et al. 2001). This feedback loop contributes to the 'switch-like' activation of CDK activity at the G1/S phase transition. A second phosphorylation mechanism regulates p27 binding to cyclin D-CDK4, where it can act as either a bound inhibitor, or a bound non-inhibitor (Ray et al. 2009). As a bound non-

inhibitor, p27 is phosphorylated on Tyr88, and helps the formation of cyclin D-CDK4 complexes and the translocation of cyclin D1 to the nucleus in G1 phase. In this conformation, CAK phosphorylation of CDK4 on Thr172 is possible and the complex can be activated. When p27 is unphosphorylated on Tyr88, however, such as in contact-arrested cells, it simultaneously occludes the active site of CDK4, and prevents the activating CAK phosphorylation, leaving the cyclin D-CDK4 complexes inactive (Soos et al. 1996; LaBaer et al. 1997; Cheng et al. 1999; Alt et al. 2002; Olashaw et al. 2004; Ray et al. 2009). It is thought that p27 binding to cyclin D-CDK4/6 complexes titrates it away from cyclin E/A-CDK2 (Cheng et al. 1998; Perez-Roger et al. 1999), but since it binds cyclin D-CDK4/6 constitutively, the extent of this titration depends on the levels of cyclin D complex available. For example, stimulating quiescent cells with mitogen induces cyclin D production, which simultaneously allows formation of cyclin D-CDK4 complexes and removal of p27 from cyclin E-CDK2.

The total levels of p27 available are regulated in other ways. These include proteolysis (as described above), increased expression in response to contact inhibition or mitogen withdrawal, particularly by translation from an internal ribosome entry site (Millard et al. 2000; Miskimins et al. 2001; Kullmann et al. 2002; Bagui et al. 2009), and altered nuclear versus cytoplasmic localisation. Localisation has been shown to depend on both Akt and Ras signalling. Phosphorylation by Akt in p27's nuclear localisation domain at Thr 157 favours export from the nucleus to the cytoplasm (Liang et al. 2002; Shin et al. 2002; Viglietto et al. 2002a). Phosphorylation of p27 on Ser10 via Ras also promotes its nuclear export and reduces its assembly into cyclin-CDK complexes (Besson et al. 2006). Cytoplasmic mislocalisation of p27 has been revealed as a mechanism of increasing cyclin-CDK2 activity in cancer cells, since the inhibition of CDK2 complexes takes place in the nucleus (Viglietto et al. 2002b; Chu et al. 2008). Low p27 levels in the nucleus have been found to correlate with poor prognosis in a range of cancers (Singh et al. 1998; Sgambato et al. 1999; Hurteau et al. 2001; Psyrri et al. 2005). This misregulation has gone some way to explaining why loss or mutation of p27 is rare in cancer (Kawamata et al. 1995). Germline disruption of p27 in mice results in

hyperplasia, increased body size, and tumour formation, particularly where the CDK-inhibitory function is specifically disrupted (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Besson et al. 2007). Oncogenic activity has been directly linked to p27 inactivation and turnover via SCFSkp2, which also removes inhibition from cyclin-CDK2 complexes normally targeted by Cip/Kip inhibitors (Kawada et al. 1997; Grimmler et al. 2007). p27 and p21 are normally upregulated in cells detached from the substratum, just as they are in mitogen-starved cells (Fang et al. 1996; Zhu et al. 1996), and hence reduced p27 levels have been implicated in anchorage independent proliferation of cancer cells (Chen et al. 1996b; Kawada et al. 1998). The role of p27 in maintaining anchorage dependence is a major theme of this thesis, and the role of anchorage signals in cell cycle control is further described in section 1.2.

1.1.2.5 How cyclin-CDK activity drives the cell cycle

From inhibitor experiments and a large number of mouse knockout models (Figure 1- 4), it is clear that cyclin-dependent kinase activity is essential for cell division, although individual cyclins and CDKs can substitute for one another, and expression of just one CDK is sufficient for proliferation (Santamaría et al. 2007). The classical view, of cyclin D-CDK4/6 initiating the cell cycle, cyclin E-CDK2 and cyclin A-CDK2 regulating S phase, and cyclin A-CDK1 and cyclin B-CDK1 triggering mitosis, came in most cases from blocking antibody or antisense DNA injection experiments. Injection of anti-cyclin D blocked cells in G1 phase and did not affect those later in the cell cycle (Baldin et al. 1993; Quelle et al. 1993). Injection of anti-cyclin E, anti-cyclin A or anti-CDK2 antibodies or antisense, but not anti-cyclin B, all prevented S phase entry, at later points than the requirement for cyclin D (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Tsai et al. 1993; Ohtsubo et al. 1995). The requirement for cyclin D, but not cyclin E, was found to be bypassed by inactivation of the Rb protein, placing cyclin D-CDK4/6 activity ahead of cyclin E-CDK2 activity in the cell cycle (Lukas et al. 1994; Tam et al. 1994; Ohtsubo et al. 1995). Following cell-cycle entry from quiescence, cyclin E-CDK2 was required to load MCM helicases onto preinitiation complexes at DNA replication origins, while cyclin A-CDK2 was required to activate DNA

Phenotypes of mice lacking cyclins/CDKs		
Genes disrupted	Phenotype	References
Cyclin D1	Viable, no embryonic lethality; minor developmental abnormalities; small size	(Fantl et al. 1995; Sicinski et al. 1995)
Cyclin D2	Viable; impaired female fertility, postnatal cerebellar development and expansion of B lymphocytes	(Sicinski et al. 1996)
Cyclin D3	Viable; impaired lymphocyte maturation	(Sicinska et al. 2003)
Cyclins D2 and D3	Death @ E18.5; hypoplastic retinas; megaloblastic anaemia	(Ciemerych et al. 2002)
Cyclins D1 and D2	Postnatal death; cerebellar abnormalities	(Ciemerych et al. 2002)
Cyclins D1, D2 and D3	Death @ midgestation (E16.5); defects in heart development; haematopoietic stem cell problems; MEFs defective in entry to and exit from quiescence	(Kozar et al. 2004)
Cyclins E1 and E2	Death @ E11.5 due to defects in endoreduplication of placental trophoblast cells (viable and healthy with either E1 or E2 alone); MEFs unable to enter S phase from quiescence	(Geng et al. 2003; Parisi et al. 2003)
Cyclin A2	Early embryonic lethality (@E5.5)	(Murphy et al. 1997)
CDK4	Viable; small size; sterile; diabetic due to lack of pancreatic islet cells; MEFs defective in cell cycle re-entry following quiescence and senesce rapidly in culture	(Rane et al. 1999; Tsutsui et al. 1999; Zou et al. 2002)

Phenotypes of mice lacking cyclins/CDKs continued		
Genes disrupted	Phenotype	References
CDK6	Viable; defects in haematopoietic compartment	(Malumbres et al. 2004)
CDK4 and CDK6	Die in utero; failure of haematopoiesis/megaloblastic anaemia; MEFs defective in cell cycle entry after quiescence and senesce rapidly in culture; form compensatory cyclin D2-CDK2 complexes with Rb kinase activity	(Malumbres et al. 2004)
CDK2	Viable up to 2 years; sterile; MEFs defective in exit from quiescence and S phase entry	(Berthet et al. 2003; Ortega et al. 2003)
CDK2 and CDK4	Embryonic lethality @E15; thin-walled heart; MEFs have decreased proliferation rate, Rb hypophosphorylation, senesce prematurely and show reduced E2F target expression (CDK1, cyclin A).	(Berthet et al. 2006)
CDK1	Fail to develop even to blastocyst stage	(Santamaría et al. 2007)
CDK2, CDK3, CDK4 and CDK6	Develop to midgestation (E12.5); some liver apoptosis; thin-walled heart; reduction in haematopoiesis; MEFs proliferate with an extended cell cycle; CDK1 binds to all cyclins	(Santamaría et al. 2007)

Figure 1- 4: Phenotypes of mice lacking various cyclins and CDKs

Table adapted from Sherr and Roberts 2004. Note that CDK1 is the only one absolutely required for cell division. In the case of cyclin isoforms, e.g. cyclins D2 and D3, the phenotypes reflect the normal tissue expression patterns: affected organs are those that principally express a single cyclin type.

synthesis at assembled replication complexes (Coverley et al. 2002). Microinjection of anti-cyclin A also prevented entry into mitosis, at a time when it was observed to bind and activate CDK1 (Pagano et al. 1992). Accumulation of cyclin B was shown to be required for mitotic entry in cell-free extracts of *Xenopus* eggs (Minshull et al. 1989).

Though studies of cyclin-CDK complexes have revealed multiple mechanisms regulating their activity, discoveries of CDK-phosphorylated targets that have a direct effect on cell cycle progression have been relatively few. Most direct CDK targets are of regulatory proteins, such as Rb (Kato et al. 1993). CDK4/6 targets Rb through binding to the LxCxE amino acid motif on cyclin D, while CDK2 preferentially phosphorylates Rb on different sites, leading to its hyperphosphorylation and inactivation (Knudsen and Wang 1996; Lundberg and Weinberg 1998; Harbour and Dean 2000). Another CDK2 and CDK4 cell cycle -regulatory target is the transcription factor Smad3, whose antiproliferative activity is inhibited by CDK phosphorylation (Matsuura et al. 2004). CDK2 also has targets more directly involved in cell cycle progression, including those regulating centrosome duplication, histone transcription, and DNA replication initiation, where CDK activity causes firing of replication origins (Krude et al. 1997; Hua and Newport 1998; Ma et al. 2000; Okuda et al. 2000; Zhao et al. 2000; Chen et al. 2002; Coverley et al. 2002). Targets for CDK1 phosphorylation in mitosis include lamins, to induce nuclear envelope breakdown, stathmin, a regulator of mitotic spindle formation, and condensin, to induce chromosome condensation (Ward and Kirschner 1990; Marklund et al. 1996; Kimura et al. 1998). The phosphorylation targets of the CDKs are still incompletely known, as hundreds of proteins contain CDK consensus sites and many of these have yet to be validated as functional CDK targets in vivo. CDK1, for example, has more than 70 substrates (Ubersax et al. 2003; Blethrow et al. 2008).

1.1.3 Cell cycle checkpoints

The effect of the complex network of regulatory mechanisms surrounding cyclin-CDK activity is to produce several ‘checkpoints’ at key junctures in the cell cycle, where it is crucial that events are correctly timed in

order to maintain smooth cell cycle progression and the stability of the genome. At these points, cell cycle progression is paused while several signals indicating the completion of previous processes are integrated. Once all the necessary cues are present, the cell is ready to go on to the next stage and the 'wait' signal is removed, allowing continuation of the cycle. If there is a problem and the checkpoint cannot be passed, the cell may either remain arrested at the previous stage, or undergo apoptosis (Elledge 1996). The mammalian cell cycle is controlled predominantly at three main checkpoints.

1.1.3.1 The Rb-E2F checkpoint

The Rb-E2F checkpoint, in G1 phase, ensures that cells have received appropriate pro-proliferative signals before committing to a full cell cycle. This was classically known as the "restriction point", roughly equivalent to START in the yeast *S. cerevisiae* (Hartwell et al. 1974; Pardee 1974). Instead of being dependent on cell size and nutrient availability to trigger cell cycle initiation, as in yeast, mammalian cells respond to cues from mitogens and from the surrounding environment, notably anchorage signals via integrins (Assoian and Schwartz 2001). Removal of mitogen signals in early G1 results in a G1 arrest, which is reversible upon reintroduction of the signal. This arrest is caused by a lack of cyclin D, which is an unstable protein sensitive to the removal of mitogens (Matsushime et al. 1991; Diehl et al. 1997). If mitogens are removed after the cell cycle has passed the restriction point, however, the cell will go on to complete one entire cycle and then both daughter cells will arrest once they return to G1 (Temin 1971; Zetterberg and Larsson 1985). The checkpoint is controlled by the Rb family of tumour suppressor proteins, which bind and repress the class of transcription factors known as E2F (Figure 1- 5). Rb repression is mediated both by direct binding and inhibition of E2F family transcription factors, and by the recruitment of histone deacetylase (HDAC1) to some promoters, which modifies the chromatin to an inaccessible state (Brehm et al. 1998; Luo et al. 1998). Integration of pro-proliferative signals results in phosphorylation of Rb and its dissociation from E2F, allowing transcription of genes required for progression into S phase, including cyclin E, cyclin A and

components of the pre-replication complex (Chellappan et al. 1991; Schulze et al. 1995; Geng et al. 1996; Yan et al. 1998; Ohtani et al. 1999).

The initial phosphorylation of Rb is carried out by cyclin D-CDK4, which prevents Rb acting as a repressor. Once cyclin E genes have been derepressed by this partial inhibition of Rb, cyclin E-CDK2 complexes will further phosphorylate the Rb protein, leading to the 'hyperphosphorylated' form, which dissociates from E2F (Knudsen and Wang 1997; Lundberg and Weinberg 1998; Harbour et al. 1999; Rubin et al. 2005). The activation of cyclin E-CDK2 complexes results in further E2F-mediated cyclin E transcription, in a positive feedback loop (Figure 1- 5). E2F also activates its own transcription (Neuman et al. 1994). Once cyclin A-CDK2 complexes have been assembled, they may maintain Rb in the hyperphosphorylated state to drive cells through S phase, but also act to release E2F from DNA and switch off the S phase initiation signal (Dynlacht et al. 1994; Krek et al. 1994; Sherr 1996). Expression of cyclin D is crucial for beginning this process of Rb inactivation, and this in turn requires a strong, sustained activation of the mitogen-activated protein kinase (MAPK) pathway by a combination of mitogen binding to receptors at the cell surface and integrin adhesion to extracellular matrix (ECM) proteins (Chen et al. 1994; Weber et al. 1997b; Roovers et al. 1999; Assoian and Schwartz 2001). Once Rb is hyperphosphorylated and no longer requires cyclin D-CDK4/6 activity, Rb inactivation becomes independent of mitogen stimulation, and the cell cycle progresses through the checkpoint (Sherr 1996).

1.1.3.2 The DNA replication checkpoint and response to DNA damage

The second major checkpoint mechanism in the cell cycle monitors DNA replication, ensuring the genome has been completely and accurately copied before the cell enters mitosis (Hartwell and Weinert 1989). Since uninterrupted DNA replication requires intact DNA, this monitoring system also responds to DNA damage, even before it can impede replication fork progression (Kastan et al. 1991; Di Leonardo et al. 1994). In mammals, evidence of a system to detect DNA breaks was first observed in cells from

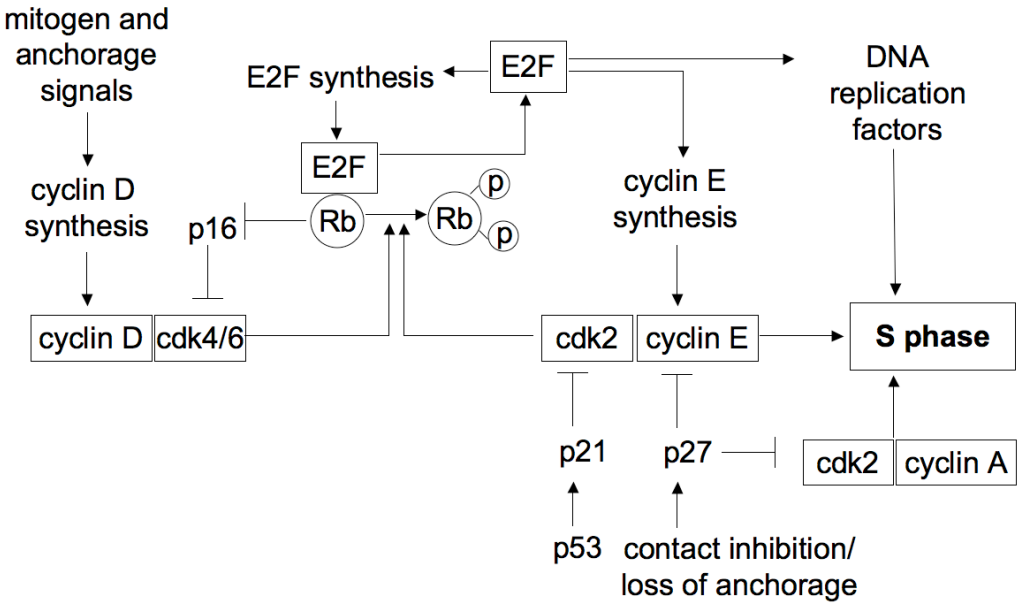


Figure 1- 5: The Rb-E2F checkpoint.

Diagram showing the Rb-E2F checkpoint mechanism linking mitogen and anchorage signalling input through G1 phase to S phase entry. Note the feedback loops: (a) negative feedback involving cyclin D-CDK4/6 activity, Rb and p16; (b) E2F release activating its own transcription; and (c) cyclin E-CDK2 activity releasing E2F to activate further cyclin E transcription and Rb hyperphosphorylation. Adapted from Zetterberg et al. 1995.

patients with ataxia telangiectasia, which fail to arrest in response to DNA damage from x-ray irradiation (Taylor 1978; Painter and Young 1980). Work in yeast and in mammalian cells has since expanded the role of the 'checkpoint' to encompass not only the sensing of DNA damage and the implementing of cell cycle arrest, but also the activation of DNA repair and recovery mechanisms (Zhou and Elledge 2000). In fact, this mechanism does not 'check' the cell at a single point in the cell cycle, but instead is activated as needed throughout G1, S and G2 phases, an indication of the importance of detecting breaks in DNA before cell division makes them irreparable and parts of the genome are lost. Thus, in addition to monitoring DNA replication, the DNA damage response plays an important role in maintaining genome stability (Hartwell 1992), as discussed in section 1.4.

In G1 and G2 phases, cell cycle arrest in response to DNA damage involves the key transcription factor p53. This protein was first discovered bound to the viral oncoprotein LT in SV40-transformed cells (Lane and Crawford 1979; McCormick et al. 1981) and later came to be known as the 'guardian of the genome' for its central tumour-suppressive role in the DNA damage checkpoint (Kastan et al. 1992; Lane 1992). p53 is stabilised in response to both ultraviolet and ionising radiation insults, and mediates cell cycle arrest via induction of the CDK inhibitor p21 (Maltzman and Czyzyk 1984; Lu and Lane 1993; Bunz et al. 1998). It is also required to induce apoptosis in response to irreparable damage (Lowe et al. 1993). The upstream 'sensors' of DNA damage, whether inflicted by exposure to environmental mutagens or by errors in DNA replication, are the kinases ATM and ATR, which in turn phosphorylate and activate the 'effector kinases' Chk1 and Chk2 (Chaturvedi et al. 1999; Liu et al. 2000; Matsuoka et al. 2000; Zhao and Piwnica-Worms 2001)(Figure 1- 6). Both Chk1 and ATR kinases are essential for development, as shown by early embryonic lethality in Chk1 and ATR-deficient mice (Brown and Baltimore 2000; de Klein et al. 2000; Liu et al. 2000; Takai et al. 2000). ATM tends to respond to double-strand breaks in DNA, and is recruited by the Mre11/Rad50/Nbs1 complex (Petrini 2000), while ATR is recruited to stalled replication forks, and other damage which exposes single-stranded DNA, by its partner ATRIP (Cortez et al. 2001; Zou and Elledge

2003). Chk1 and Chk2 are recruited via interaction with adaptor proteins such as Claspin (Kumagai and Dunphy 2000). In G1 phase, phosphorylation by Chk1 and Chk2 stabilises p53 to induce cell cycle arrest (Chehab et al. 1999; Chehab et al. 2000; Hirao et al. 2000; Shieh et al. 2000). ATM and ATR may also phosphorylate p53 directly (Khanna et al. 1998; Hall-Jackson et al. 1999). In G2 phase, Chk1 is activated by BRCA1 in response to DNA damage, and stabilises securin, thus preventing separation of chromosomes until DNA has been repaired (Yarden et al. 2002; Agarwal et al. 2003). Chk1 and Chk2 also directly inhibit CDC25, and Chk1 activates Wee1, preventing the activation of cyclin-CDK complexes (Sanchez et al. 1997; Blasina et al. 1999; Lee et al. 2001). For example, Chk1 keeps cyclin B-CDK1 inactive when activated by ATR in response to unreplicated DNA, to prevent mitotic entry with an improperly replicated genome (Guo et al. 2000; Hekmat-Nejad et al. 2000; Lee et al. 2005).

Although S phase is not initiated while DNA damage is present, a DNA damage response may be activated during S phase, in response to inhibition of replication (leading to replication fork stalling), or errors in DNA synthesis (Zhou and Elledge 2000; Lopes et al. 2001; Tercero and Diffley 2001). When induced by environmental factors or oncogenes, this S phase inhibition is known as replication stress (Desany et al. 1998; Osborn et al. 2002). The induction of replication fork stalling and the DNA damage response by oncogenes will be discussed in section 1.3. Intra-S phase damage does not result in immediate global arrest, but S phase progression is slowed, as the DNA damage response prevents firing of late replication origins (Santocanale and Diffley 1998; Heffernan et al. 2002). The response also prevents entry into mitosis, via activation of Chk1, as described above. In order to maintain the activation of the checkpoint in regions of damaged DNA, the histone variant H2A is phosphorylated by ATM or ATR to produce the marker known as γ-H2AX (Rogakou et al. 1999; Burma et al. 2001; Fernandez-Capetillo et al. 2002; Furuta et al. 2003; Shroff et al. 2004). Although not required for initiation of the checkpoint (Celeste et al. 2003), γ-H2AX interacts with adaptor proteins such as MDC1 to increase recruitment of other DNA damage proteins and amplify the response (Stucki et al. 2005). The ‘spreading’ of the signal

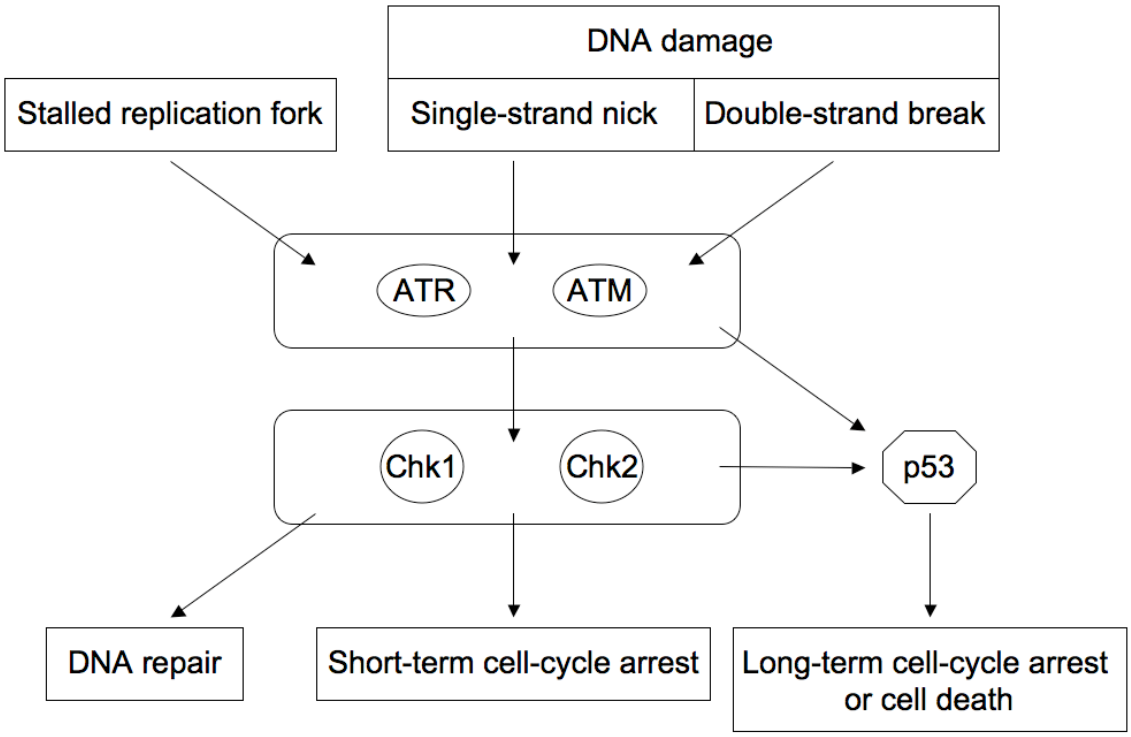


Figure 1- 6: The DNA damage response.

Overview of the DNA damage response, involving recruitment of the 'sensor kinases' ATM or ATR to the damage site, and activation of the 'effector kinases' Chk1 and Chk2 to produce either DNA repair, cell-cycle arrest, or p53-induced cell death. See text for more details, and references. Diagram adapted from Morgan 2007.

along DNA allows recruitment of cohesins to assist repair by sister-chromatid exchange in G2 phase (Ström et al. 2004; Unal et al. 2004). Once repaired, the checkpoint is alleviated by dephosphorylation of key proteins such as p53 and Chk1, mediated by phosphatases such as PPM1D (Lu et al. 2005). Coordination of this checkpoint by proteins located at the replication fork also ensures complete and proper DNA replication, as the presence of a fork in itself means that replication has not finished (Tercero et al. 2003).

1.1.3.3 The spindle assembly checkpoint

The final cell cycle checkpoint acts during mitosis, to ensure that the sister chromatids are equally segregated to the daughter cells so that each cell inherits a complete set of chromosomes. The spindle assembly checkpoint (SAC) verifies that all chromatids are attached to the spindle at the kinetochore by microtubules, and are under tension, before anaphase is triggered and cohesin is cleaved. The SAC mechanism (Figure 1- 7) senses unattachment of kinetochores to the microtubules and responds to the tension produced when both chromatids are securely tethered to opposite spindle poles and ready to be separated (Waters et al. 1998; Shannon et al. 2002). While the SAC is active, the APC does not recognise cyclin B or securin, and so anaphase is inhibited, thus preventing unequal segregation of chromosomes and aneuploidy (Musacchio and Salmon 2007). The components of the SAC were first discovered in yeast, from mutants defective in mitotic arrest (Hoyt et al. 1991; Li and Murray 1991), and these are conserved throughout the eukaryotes. A mitotic checkpoint complex (MCC) composed of Mad2, BubR1, Bub3 and the APC activator Cdc20 was proposed to mediate the SAC effector signal at kinetochores (Sudakin et al. 2001). By laser ablation experiments, a single unattached kinetochore was shown to be sufficient to activate the checkpoint (Rieder et al. 1995). The kinase Aurora B was shown to target BubR1 and Mad2 to kinetochores, and to correct improper microtubule attachments, for example having both sides of the kinetochore connected to the same spindle pole (Ditchfield et al. 2003; Morrow et al. 2005; Tanaka et al. 2005).

The key target of the SAC mechanism is the APC activator Cdc20, and overexpression of Cdc20 was shown to be sufficient to overcome the checkpoint

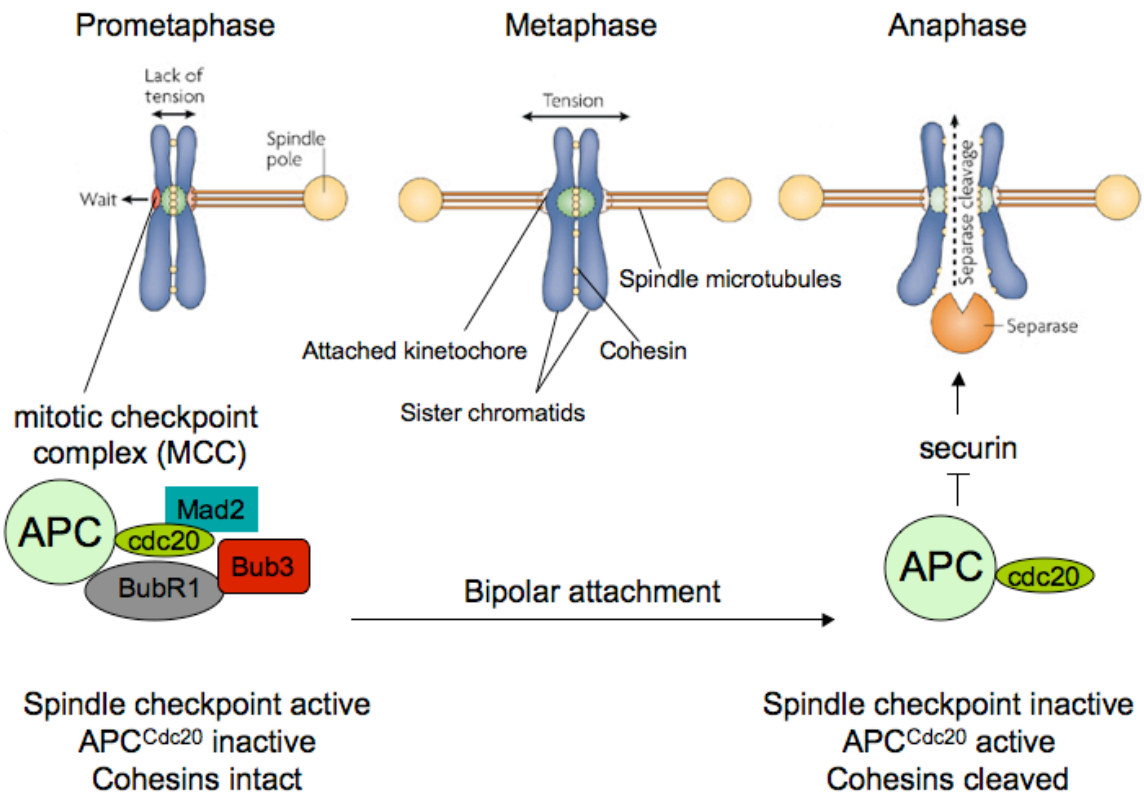


Figure 1- 7: The spindle assembly checkpoint.

Cartoon shows a mitotic chromatid pair (blue) in the process of being captured by spindle microtubules (yellow). In prometaphase (left), Mad2 levels at unattached kinetochores are high, and Cdc20 is targeted for degradation, generating a 'wait' signal. The bioriented attachment of chromosomes at metaphase (centre) results in application of tension to the kinetochores and inactivates the spindle checkpoint 'wait' signal. Checkpoint inactivation triggers anaphase (right): activation of APC^{Cdc20} and separation of the sister chromatids by cohesin cleavage. See text. Figure adapted from Musacchio and Salmon 2007.

(Hwang et al. 1998; Kim et al. 1998; Pan and Chen 2004). However, it was unclear whether the MCC acted to sequester Cdc20, or inhibit it. It was also suggested that the SAC is turned off by disruption of the MCC following APC-mediated ubiquitination of Cdc20 (Reddy et al. 2007). Recently, a new study (Nilsson et al. 2008) has challenged this idea, with data suggesting that Mad2 only transiently associates with the MCC, and instead it may collaborate with BubR1 and Bub3 to 'present' Cdc20 to the APC for ubiquitination. Their results also suggest that, far from turning the SAC signal off, Cdc20 ubiquitination by the APC acts to maintain the checkpoint by destroying the subunit responsible for recognition of cyclin B and securin. This would prevent APC activation and separation of the chromosomes until the checkpoint has been deactivated and Cdc20 is able to associate with the APC normally (Nilsson et al. 2008).

1.1.3.4 Other regulatory mechanisms: DNA licensing

Early cell fusion experiments demonstrated that G1 nuclei are competent, or 'licensed' for DNA replication, and S phase nuclei can promote early DNA synthesis in G1; but that G2 nuclei are not competent to replicate and are refractive to S phase activation (Rao and Johnson 1970; Stillman 1996). This DNA 'licensing' mechanism ensures that each strand is replicated exactly once per cell cycle (Blow and Dutta 2005). Either incomplete replication or rereplication of any portion of DNA leads to improper separation of the daughter strands, so that chromosomes are likely to break at mitosis, resulting in genome instability and possible initiation of a fatal cycle of chromosome breakage and fusion (see section 1.4). To prevent this, DNA replication initiation has a complex 'firing' mechanism, which requires the recruitment and co-operation of several proteins at replication origins (Figure 1- 8). The pre-replication complex (pre-RC) consists of the origin recognition complex (ORC), the minichromosome maintenance (MCM) helicase complex, and the Cdc6 and Cdt1 regulatory proteins. Both MCM subunit and Cdc6 genes are targets of E2F regulation, and their expression is therefore cell-cycle regulated (Yan et al. 1998; Ohtani et al. 1999). The pre-RC is assembled on DNA only during late M

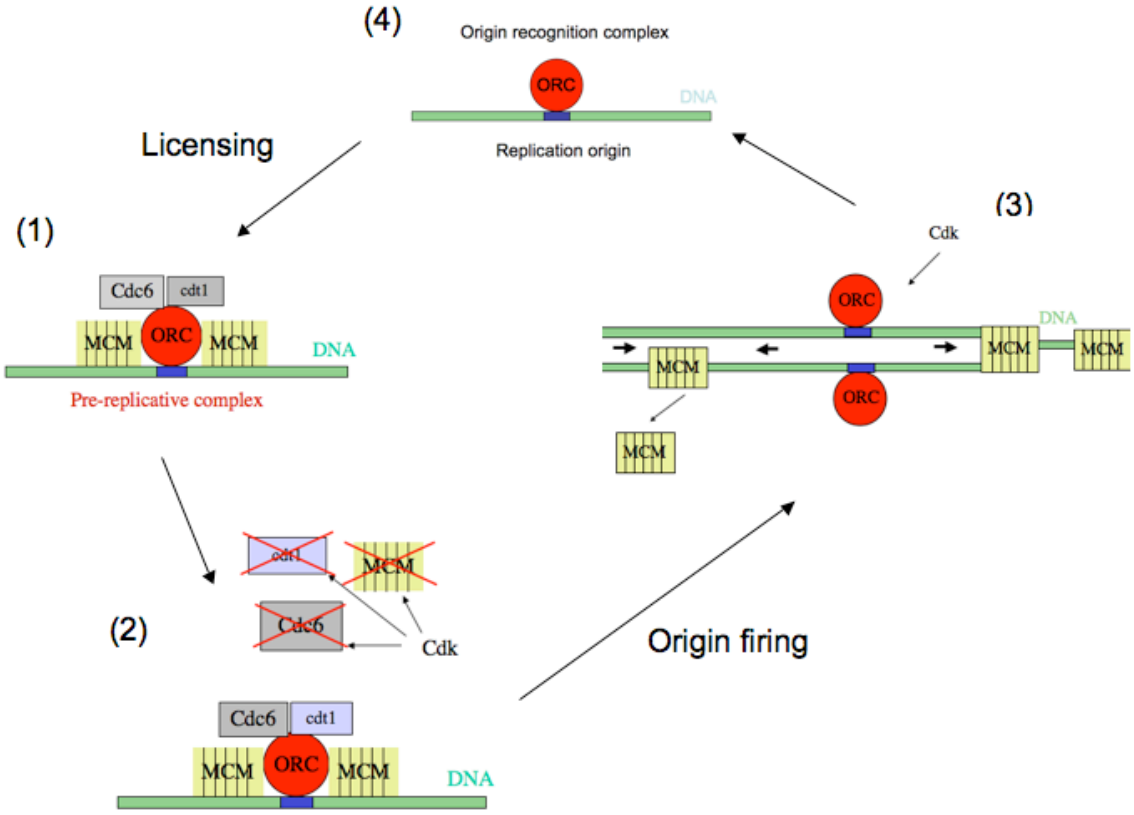


Figure 1- 8: DNA replication licensing.

(1) In late M and early G1 phase, CDK activity is low and pre-replicative complexes (pre-RC) assemble at replication origins.

(2) As CDK activity rises in G1 phase, pre-RC components are targeted for removal, either by proteolysis or by nuclear export, so that no further licensing can occur.

(3) High CDK activity triggers origin firing, marking the start of S phase. MCM helicases move away from replication origins with the replication fork in both directions, leaving the origin unlicensed. As DNA replication ends, replication forks meet and MCMs are displaced from DNA.

(4) During G2 and early M phases, CDK activity remains high, thus inhibiting further replication licensing until the genome has segregated and the next cycle begins. See text for references.

and early G1 phase, when cyclin-CDK activity is low (Blow and Hodgson 2002; Nishitani and Lygerou 2002). CDKs and cdc7, which forms a cyclin-CDK-like complex with Dbf4p (ASK), then recruit DNA replication enzymes to the complex and trigger initiation of DNA replication - the 'firing' of replication origins (Sclafani 2000; Walter and Newport 2000; Zou and Stillman 2000; Masai and Arai 2002; Devault et al. 2008). Elongation requires MCM helicase activity, so the MCM complex moves along with the replication fork, leaving the origin once more unlicensed (Aparicio et al. 1997; Labib et al. 2000; Shechter et al. 2004). Cdt1 protein is necessary for licensing, but it is bound and inactivated by the replication inhibitor Geminin during S and G2, so that origin licensing is inhibited during this time (Hodgson et al. 2002; Lee et al. 2004). Cdt1 is also degraded in S and G2 phases by the proteasome, to prevent re-licensing of replication origins before mitosis (Li and Blow 2005; Nishitani et al. 2006). Geminin is destroyed by the APC in late mitosis, releasing Cdt1 to allow the next round of origin licensing (McGarry and Kirschner 1998; Ballabeni et al. 2004).

The reliance of origin 'firing' and licensing on high and low CDK activity respectively ensures that origins cannot be licensed and 'fired' at the same time, and restricts licensing to one short period per cell cycle so that DNA is replicated only once (Blow and Dutta 2005). In yeast, CDK activity normally inhibits rereplication by phosphorylating the origin recognition complex, by downregulating cdc6 activity and by excluding MCM proteins from the nucleus (Nguyen et al. 2001; Liku et al. 2005). In metazoans, CDK activity regulates chromatin binding of the pre-RC components to control licensing (Findeisen et al. 1999). CDK phosphorylation of cdt1 also leads to its proteasomal degradation (Sugimoto et al. 2004).

Inhibition of CDK activity can lead to rereplication and genome instability (Itzhaki et al. 1997; Bates et al. 1998; Machida and Dutta 2007; Porter 2008). Removal of geminin-mediated Cdt1 inhibition also leads to rereplication, and this also triggers the DNA damage checkpoint (Mihaylov et al. 2002; Melixetian et al. 2004; Zhu et al. 2004; Davidson et al. 2006; Gonzalez et al. 2006a; Zhu and Dutta 2006; Kerns et al. 2007). Endoreduplication cycles, where the genome is repeatedly replicated without intervening mitoses, are

required in specialised cell types such as placental trophoblast cells and must be carefully controlled. Initiation of endoreduplication requires a decrease in cyclin A/B-CDK1 activity, either by inhibiting the CDK or decreasing expression of the cyclin (Coverley et al. 1998). The endocycles themselves are characterised by an oscillation of cyclin E-CDK2 activity (Lilly and Spradling 1996; MacAuley et al. 1998; Hattori et al. 2000). Consistent with the expression of replication licensing factors being characteristic of proliferating cells, inappropriate overexpression of proteins such as cdc6 and Cdt1 can be seen in cancer cells, and may contribute to driving their proliferation (Arentson et al. 2002; Karakaidos et al. 2004; Gonzalez et al. 2006b; Lau et al. 2007). Rb loss, frequently seen in cancer cells, is also associated with overreplication, and is thought to promote association of replication factors with chromatin under nonpermissive conditions (Niculescu et al. 1998; Srinivasan et al. 2007).

1.2 Signals from anchorage

1.2.1 Integrins and the extracellular matrix

Physical attachment of cells to the extracellular matrix (ECM) is mediated by integrins, as well as other glycoprotein receptors for ECM, such as syndecans and CD44 (Buck and Horwitz 1987; Saunders et al. 1989; Aruffo et al. 1990; Morgan et al. 2007). Cell-matrix adhesion is fundamental to determining cell behaviour, and anchorage signals provide the cell with information about the tissue microenvironment as well as input into controls for proliferation, differentiation, and survival (Adams and Watt 1989; Meredith et al. 1993; Assoian 1997; Lukashev and Werb 1998). Integrins, for example, can sense the composition of the surrounding matrix and actively signal to the cell via proteins associated with the integrin cytoplasmic domain (Geiger et al. 2001). Integrins are structured as protein heterodimers of one alpha and one beta chain (Buck and Horwitz 1987). 24 heterodimer combinations of the 18 alpha and 8 beta subunits are known in human cells, and these have different ligand-binding specificities (Takada et al. 2007). The beta chain has a longer cytoplasmic tail than the alpha, containing the protein-protein interaction motifs NXXY and NPXY, which bind the cytoskeleton-associated proteins talin and paxillin (Mitra and Schlaepfer 2006).

A key property of integrins is mechanotransduction of physical information, such as stiffness and tension, into biochemical signals within the cell, by modifying their structural conformation in response to applied forces (Zhu et al. 2008). They are ideally placed for this role, acting as anchor points at the cell membrane for the cytoskeleton, in clusters of protein known as focal adhesions (Burridge et al. 1988; Geiger et al. 2009). Focal adhesion complexes also interact with mitogen receptor tyrosine kinases (Figure 1- 9). Within the protein clusters is a vast concentration of signalling molecules, among them the kinases FAK (focal adhesion kinase) and ILK (integrin-linked kinase) (Miyamoto et al. 1995; Plopper et al. 1995; Li et al. 1999). Both these and the integrins themselves interact with adaptor proteins to affect signalling by the Rho, Rac, PI3K and MAPK pathways, influencing cell migration, proliferation and apoptosis (Schaller et al. 1994; Schlaepfer et al. 1994; Frisch et al. 1996; Wary et al. 1996; Lin et al. 1997; Clark et al. 1998; D'Amico et al. 2000).

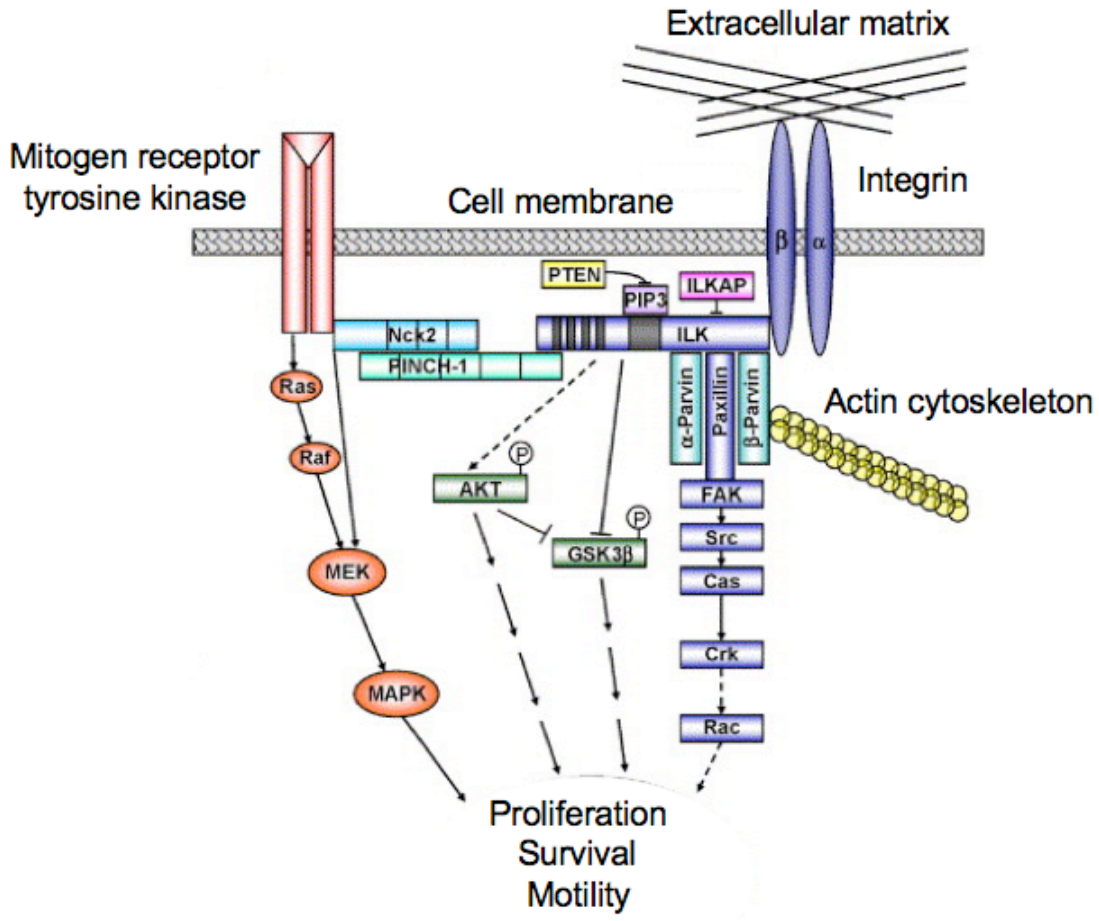


Figure 1- 9: Signalling downstream of integrins at focal adhesions.

Cartoon shows the interaction of signalling proteins with integrins, including integrin-linked kinase (ILK) and focal adhesion kinase (FAK), with some of the adaptor proteins linking them to receptor tyrosine kinases and the cytoskeleton. An indication of the signalling pathways downstream of the kinases (Ras/MAPK, Akt, Rac, etc.) is shown in simplified form. Adapted from Hehlgans et al. 2007.

The stiffness and composition of the extracellular matrix is known to affect the morphology and differentiation of attached cells (Garcia et al. 1999; Discher et al. 2005; Yeung et al. 2005; Engler et al. 2006), by altering the tension applied to the elastic cytoskeletal network and the signalling from focal adhesions (Chen et al. 1997; Chen et al. 2003a; Ingber 2003). Tumour microenvironments are often less pliable than normal tissue, which has been suggested to contribute to altering the integrin expression profile and the consequent change in signalling observed in cancer cells (Paszek et al. 2005). The presence of certain extracellular matrix components, such as fibronectin, or expression of the fibronectin receptor $\alpha5\beta1$ can inhibit invasion and metastasis, while expression of promiscuous integrins such as $\alpha v\beta3$ can promote migration of cells on many different extracellular matrix proteins and encourage angiogenesis (Varner and Cheresh 1996). Cells may also modify their microenvironment by secreting their own ECM components and matrix-degrading enzymes, thus influencing the behaviour of surrounding cells as well as their own: a situation that is particularly relevant in tumours (Kubo et al. 1984; Chernousov et al. 1996; Liotta and Kohn 2001; Phillips et al. 2003; Larsen et al. 2006).

1.2.2 Detachment: Arrest or anoikis?

When detached from the extracellular matrix, normal mammalian cells may respond in several ways: either programmed cell death, known as anoikis, in epithelial or endothelial cells; differentiation, in keratinocytes; reversible cell cycle arrest, in mesenchymal and fibroblastic cell types; and in transformed or cancerous cell types, cells continue to proliferate (Stoker et al. 1968; Otsuka and Moskowitz 1975; Green 1977; Frisch and Francis 1994). Most proliferating cells cell-cycle arrest following detachment from the ECM in the G1 phase of the cell cycle, at the classical Rb/E2F checkpoint, due to insufficient cyclin D induction (Assoian and Klein 2008). Cell-cycle arrest at G1/S phase by overexpression of CDK inhibitors allows evasion of anoikis in mammary epithelial cells (Collins et al. 2005). Anoikis occurs where the lack of integrin signalling results in insufficient activation of pro-survival pathways by PI3K, and apoptotic cell death (Khwaja et al. 1997). Signalling via PI3K and Akt mediates survival in several ways, including the phosphorylation and inhibition

of pro-apoptotic proteins such as Bad and caspase 9 (Datta et al. 1999; Khwaja 1999). Both ILK and FAK signalling downstream of integrins have been shown to suppress anoikis (Attwell et al. 2000; Sonoda et al. 2000). Loss of integrin signalling can also result in the downregulation of growth factor signalling, which contributes to anoikis, since both IGF and EGF receptor signalling promote survival (Moro et al. 1998; Valentinis et al. 1998; Reginato et al. 2003). In mesenchymal cells such as fibroblasts, growth factor signalling alone is sufficient for survival, whereas in epithelial cells, both integrin and growth factor signalling is needed (Frisch and Screaton 2001). Disruption of the cytoskeleton following loss of anchorage in epithelial cells causes release of pro-apoptotic factors from their sequestration on cytoskeletal filaments, contributing to anoikis (Puthalakath et al. 1999; Puthalakath et al. 2001). Epithelial cells also receive adhesion signals from surrounding cells via adherens junctions, mediated primarily by E-cadherin (Nagafuchi et al. 1987). Removal of E-cadherin signalling is thought to contribute to detachment-induced anoikis (Fouquet et al. 2004).

Oncogenes such as Ras allow anoikis evasion via activation of pro-survival pathways, including PI3K/Akt and Raf/MAPK, and inhibition of pro-apoptotic proteins including Bad, Bim, Bak, and Fas (Khwaja et al. 1997; Fenton et al. 1998; Rosen et al. 1998; Bonni et al. 1999; Rosen et al. 2000; McFall et al. 2001; Rong et al. 2005; Liu et al. 2006; Jin et al. 2007; Vasudevan et al. 2007; Goldstein et al. 2009). It has recently been reported that autophagy is induced following cell detachment of all kinds, and that this may promote the survival of cells rather than apoptosis (Fung et al. 2008). This discovery led review authors to speculate that autophagy may allow detached cancer cells to survive for long periods ‘dormant’, as did cells in a breast cancer model lacking β1 integrin expression (White et al. 2004; Aguirre-Ghiso 2007; Lock and Debnath 2008).

1.2.3 How anchorage signals feed into cell cycle controls

Mitogen signals alone are not sufficient to drive cell cycle progression, and a combination of anchorage and mitogenic signals is required to bypass the G1/S phase restriction point in normal adherent cells (Assoian and Schwartz

2001). This is due to the level of sustained ERK signalling needed to induce cyclin D1 transcription – if either mitogens or anchorage are missing, then the period of ERK phosphorylation is limiting (Zhu and Assoian 1995; Lavoie et al. 1996; Miyamoto et al. 1996; Renshaw et al. 1997; Weber et al. 1997a). Consistent with this, cyclin D1 transcription can be restored by constitutively activating ERKs, and ERK activation is dispensable if cyclin D1 is overexpressed (Roovers et al. 1999; Villanueva et al. 2007). As well as integrin ligation, cytoskeletal integrity and a 'spread' cell shape have been identified as essential for cell cycle progression in anchorage-dependent cells (Böhmer et al. 1996; Huang et al. 1998; Aplin and Juliano 1999).

When translated, cyclin D1 binds to CDK4/6 and this enzyme complex phosphorylates the retinoblastoma protein Rb, thus releasing E2F transcription factors from their repressed state and allowing transcription of S phase genes such as cyclin A (Weinberg 1995) (Figure 1- 10). Cyclin D1 overexpression is sufficient to promote S phase entry in suspended Rat1 cells (Resnitzky 1997). However, in the absence of anchorage, cyclin D-CDK4/6 is not induced in sufficient quantities to titrate p27 away from cyclin E-CDK2, and Rb cannot be phosphorylated by either CDK (Assoian 1997). Not all ECM components have a positive effect on cyclin D1 transcription: for example, fibronectin will promote transcription and cell-cycle progression, while high molecular weight hyaluronan antagonises mitogenic signalling and inhibits cyclin D1 induction (Kothapalli et al. 2007). This demonstrates that the cell cycle is sensitive to changes in the composition of the microenvironment, and does not respond only to its state of physical attachment. Cyclin D1 is therefore a key barometer of the total anchorage input to the cell cycle, and because the protein is so labile, levels are quick to respond to downregulation when the cell is unattached (Diehl et al. 1998).

In addition to activation of the ERK-MAPK pathway, by integrins via Rho, the focal adhesion kinase (FAK) also contributes to cell cycle signalling from integrin clusters, both by activating cyclin D1 transcription via the transcription factor KLF8 (Zhao et al. 2003) and by promoting the proteolytic destruction of p27 via the E3 ubiquitin ligase component Skp2 (Carrano et al. 1999; Bond et al. 2004). Both p21 and p27 CDK inhibitors are induced

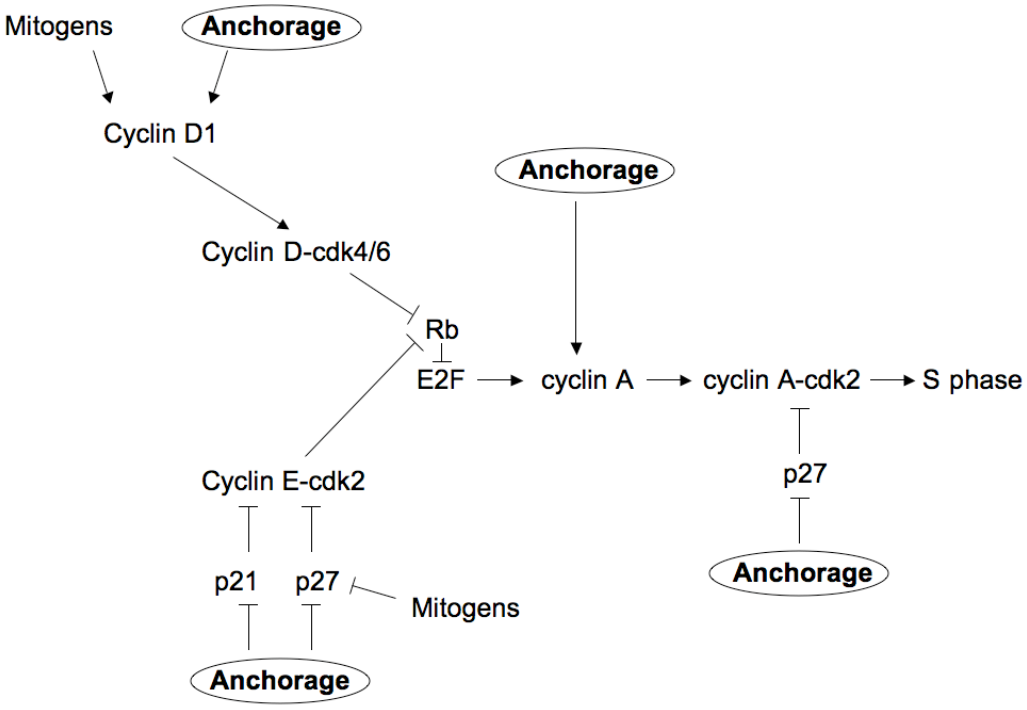


Figure 1- 10: How anchorage signals contribute to G1/S phase progression.

Signals from the extracellular matrix cooperate with mitogens to drive cells into S phase. In particular, anchorage signals are required for cyclin D1 induction at both the mRNA and protein level; downregulation of the CDK inhibitors p21 and p27; and induction of cyclin A transcription. See text for references. Diagram adapted from Assoian 1997.

following detachment and loss of integrin signalling (Zhu et al. 1996). In the case of p27, this is achieved through increased stabilisation and decreased proteolysis of the protein via control of Skp2 mRNA and protein by FAK. In the case of p21, control is again through proteasomal degradation, via Cdc42 and Rac1 signalling from integrins (Bao et al. 2002). These results explain the anchorage dependence of cyclin E-CDK2 activity, which is inhibited by p21 and p27 (Fang et al. 1996).

Cyclin A induction is another factor limiting cell cycle progression in detached cells, as this was found to be lacking, independent of cyclin D and cyclin E expression, in NRK cells, and re-expression of cyclin A was sufficient to rescue S phase entry and cell division (Guadagno et al. 1993). Cyclin A promoter activity depends on both Rb-family phosphorylation, which determines the timing of activation, and on CREB phosphorylation and binding to the cAMP response element (CRE) located in the cyclin A promoter, which regulates the extent of induction (Bottazzi et al. 2001). However, Bottazzi et al found that CREB phosphorylation depends only on mitogenic and not on anchorage signalling, so the reason why cyclin A expression was anchorage dependent in NRK cells, which already have a defective Rb checkpoint, was unclear. Kramer et al found that the binding of CCAAT-binding protein (CBP) to the cyclin A promoter correlated with cyclin A expression and was anchorage dependent in NRK cells, so this may explain the anchorage dependence of cyclin A expression seen in the later paper (Krämer et al. 1997).

1.3 Cell transformation and cancer

1.3.1 Transformation and tumourigenesis as multistep processes

Cancer has long been recognised as a heterogeneous disease, with manifold causes, including exposure to environmental carcinogens, congenital and spontaneous mutations, and viral infection. Any tissue can be affected, from skin epithelia to blood leukocytes, with the common factor being the inappropriate proliferation of cells at the expense of healthy tissue function. Malignancies are clinically subdivided into three types, according to the cell of origin: epithelial carcinomas, e.g. breast cancer; cancers in tissues of mesenchymal origin, such as connective tissue, muscle, and bone, e.g. osteosarcomas; and leukaemias and lymphomas, originating in the haematopoietic cells of the blood and lymph. Although the causative lesions vary between subtypes, there are broad parallels in the process whereby a normal healthy cell becomes cancerous and forms a tumour, known as cellular transformation and tumourigenesis. The discovery over many years of cancer-causing genetic changes has revealed that more than one modification is required to transform a cell, although often one change will increase the probability of further spontaneous mutation (Loeb et al. 1974; Clark et al. 1988; Christofori and Hanahan 1994; Kinzler and Vogelstein 1996; Loeb et al. 2008). Generally, these changes can be classified into oncogenes, which promote tumourigenesis upon activation or overexpression, and tumour suppressors, whose inactivation precipitates tumourigenesis. Occasionally a single protein turns out to have both oncogenic and tumour-suppressive properties depending on the cellular context, but these distinctions are still useful in thinking about the aetiology of cancer and the tumourigenic process.

Oncogenes were first isolated as transforming components from tumour-promoting viruses (Vogt and Dulbecco 1960; Harvey 1964; Todaro et al. 1964; Huebner and Todaro 1969; Martin 1970). The DNA from tumours was sufficient to transform normal cells, and normal cells were found to contain endogenous ‘proto-oncogenes’ related to the genes in tumour viruses (Stehelin et al. 1976; Shih et al. 1979). The Ras family of oncogenes were identified in rat sarcoma viruses and found to encode GTP-binding proteins, unlike the original viral oncogene, src, which is a tyrosine kinase (Scolnick et al. 1979).

The oncogenic mutation in bladder cancer cells was discovered to be a single amino acid change at position 12, replacing glycine with valine (Reddy et al. 1982; Tabin et al. 1982). This bladder cancer mutation was found to be in the Ras gene (Der et al. 1982; Parada et al. 1982). Cancers induced by chemical mutagens were also found to have mutant Ras, indicating oncogenic mutation as the mechanism underlying tumourigenesis in all cancers, whether from viruses, carcinogens or spontaneous lesions (Sukumar et al. 1983). The G12V activating mutation in Ras was found to decrease its intrinsic GTPase activity (Gibbs et al. 1984; McGrath et al. 1984; Sweet et al. 1984) and more importantly, prevent the stimulation of GTP hydrolysis by GTPase-activating proteins, or RasGAPs (Trahey and McCormick 1987; Adari et al. 1988). Since Ras is active in its GTP-bound form, the G12V mutation confers constitutive activation of Ras downstream effectors, including the Ras/ Raf/ MEK/ ERK MAPK pathway, and PI3K/ Akt signalling (Marshall 1996).

The first tumour suppressor gene to be defined was Rb, which was cloned following initial mapping of the locus in patients with retinoblastoma (Cavenee et al. 1983; Friend et al. 1986) and subsequently found to play a central role in the G1/S checkpoint due to its inhibition of E2F-mediated transcription (Chellappan et al. 1991; Bartek et al. 1996). The celebrated 'guardian of the genome', p53, mediator of the DNA damage checkpoint, was in fact originally thought to be an oncogene when the protein was first isolated bound to the viral oncoprotein LT (Lane and Crawford 1979; Linzer and Levine 1979). Subsequent work showed that both LT binding, and the frequent p53 mutations found in cancer cells, actually inactivated the protein, establishing it as a tumour suppressor (Baker et al. 1990; Levine et al. 1991; Lane 1992). Most tumour suppressors either directly inhibit proliferation, such as Rb ("gatekeepers"), or maintain genome stability, such as BRCA2 ("caretakers"). p53's key role in the DNA damage checkpoint means it may be regarded as both a gatekeeper, controlling cell cycle arrest and apoptosis, and a caretaker, whose function ensures the stability of the genome (Kastan et al. 1991; Lane 1992; Kinzler and Vogelstein 1997; Bunz et al. 1998).

The classical view assumes that oncogenic mutations, such as activation of Ras, are dominant, while tumour suppressive mutations, such as Rb

inactivation, are recessive, and both gene copies must be inactivated before cancer can occur. This is known as the two-hit hypothesis, and was first described following statistical analysis of retinoblastoma incidence in patients with both inherited and spontaneous Rb mutations (Knudson 1971). Subsequently, some tumour suppressor genes were found to be exceptions to this rule, since inactivation of just one allele produces a phenotype. Such genes, for example the CDK inhibitor p27, are known as haploinsufficient (Fero et al. 1998).

In cancers that characteristically show particular mutations at certain histological stages, a 'typical' process of cellular transformation and tumourigenesis can be described (Figure 1- 11). This was first carried out for colon cancer, where the inactivation of the APC (adenomatous polyposis coli), DCC (deleted in colon cancer), and p53 tumour suppressor genes together with the oncogenic activation of Ras were described as contributory to the tumourigenic process: in this case, progression from normal epithelium to a non-cancerous benign polyp through intermediate and late adenoma stages to a fully invasive and possibly metastatic carcinoma (Fearon and Vogelstein 1990).

While it may be argued that there is no 'typical' tumourigenic process, this model remains the basis for considering the mechanisms of tumourigenesis as a stepwise process. Notably, the original Vogelstein model did not specify with any rigidity the precise order of mutations; now the timing of genetic alterations in all cancer types has assumed greater importance (alongside frequency of presentation in tumours), indicating which are truly independent causative or initiating events, which are necessary to support other mutations, and which are simply incidental to the state of hyperproliferation (Kinzler and Vogelstein 1996; Buerger et al. 1999; Futreal et al. 2004; Welsch et al. 2007; Loeb et al. 2008). In the case of Ras and p53, the mutations often occur together, as oncogenic Ras is antiproliferative in the presence of a p53 checkpoint (Hirakawa and Ruley 1988; Ridley et al. 1988; Hicks et al. 1991). Together, oncogenic Ras and mutant p53 cooperate to cause a catalogue of genetic changes characteristic of transformation (Lloyd et al. 1997; McMurray et al. 2008).

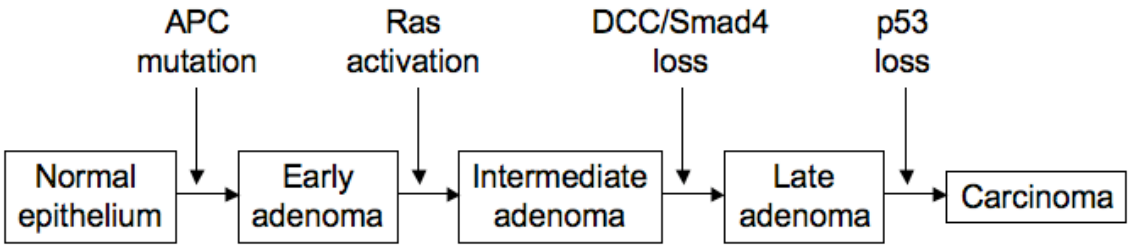


Figure 1- 11: Vogelstein's colon cancer model.

Examples of common genetic changes associated with the progression from normal colon epithelium to cancer. Colorectal cancer is especially amenable to this type of analysis, since the histological stages of tumourigenesis are well defined and many tissue samples are available for study. APC = adenomatous polyposis coli, an inhibitor in the Wnt mitogenic pathway frequently lost in both inherited and sporadic colon cancers. DCC = deleted in colon cancer, a protein involved in cell adhesion. The gene is at the 18q chromosome locus, which also contains the Smad4 gene, and is frequently lost in colon cancer. Diagram adapted from Kinzler and Vogelstein 1996.

In parallel with investigations into the causative mutations in human cancers, work in several cell lines has sought to determine the biochemical effects of and interplay between these genetic changes, studying in vitro 'transformation' as a model for in vivo tumourigenesis. From the earliest studies on the effect of tumourigenic viruses on established cell lines, with unknown mutations (Vogt and Dulbecco 1960; Todaro et al. 1964), work has moved on to using cultured cells with defined genetic changes introduced. Genetic changes can be introduced one by one, to study their effects both individually and in combination (Hahn et al. 1999; Rangarajan et al. 2004; Funes et al. 2007). These cellular models of tumourigenesis have had significant success in reproducing features of human cancers, including ovarian cancer (Liu et al. 2004b), breast cancer (Elenbaas et al. 2001), and glioma (Rich et al. 2001). The classical characteristics common to transformed cells include focus formation (proliferation of a small clone over and above a monolayer, indicating loss of contact inhibition); mitogen independence; increased motility and other morphological changes; and colony formation in soft agar (indicating loss of anchorage dependence). These and other properties are also characteristic of cancer cells, as detailed below.

1.3.2 Hallmarks of cancer: independence from extracellular cues

The reason for several changes being required to transform normal cells into a cancer is twofold: firstly, because of the multiple internal control mechanisms that regulate the behaviour of normal cells to achieve coordinated function of the tissue; and secondly, because of the reliance of normal cells on extracellular cues to drive their growth and proliferation. In order to become tumourigenic, cells must be able to divide regardless of external signals, and also escape any antiproliferative or pro-apoptotic cues. These key elements led Hanahan and Weinberg to propose the six "hallmarks of cancer" (Hanahan and Weinberg 2000):

1) Self-sufficiency in growth signals
2) Insensitivity to anti-growth signals
3) Limitless replicative potential
4) Evading apoptosis

5) Sustained angiogenesis
6) Tissue invasion and metastasis

The first four points refer to cancer at the cellular level, while the latter two refer to the maintenance and development of the tumour as a whole, and are relevant to solid-tissue cancers of epithelial and mesenchymal origin rather than haematological disorders.

1.3.2.1 Self-sufficiency in growth signals/ Insensitivity to anti-growth signals

A cell can become self-sufficient for mitogens in several ways: (a) by overproduction of the mitogens themselves, such as PDGF (Potapova et al. 1996); (b) by hyperactivation of mitogen receptors such as the EGFR (Haley et al. 1989); (c) by activation of downstream signalling proteins, such as Ras (Malumbres and Pellicer 1998); and (d) by activation of transcription factor targets of mitogen signalling, such as Myc (Pelengaris et al. 2002). Overactivation of signals earlier in the hierarchy can affect several downstream pathways, including cell growth and survival as well as proliferation. In this way, a single mutation can fulfil more than one cancer requirement at once. For example, Ras downstream targets include PI3K, which signals to promote growth via mTOR and to inhibit apoptosis, as well as the well-characterised Raf/MEK/ERK MAPK pathway promoting cell-cycle progression (Marshall 1996). Constitutive activation of oncogenes may in itself confer insensitivity to anti-growth signals, but in many cases inactivation of tumour suppressors is also required to overcome mechanisms that act to prevent inappropriate proliferation, such as cell cycle arrest, apoptosis and senescence. Enforced cell cycle progression by oncogenes can initiate a DNA damage response by causing replication stress, leading to double-strand breaks and activation of p53-dependent checkpoint mechanisms. This will result in senescence, unless p53 or Chk2 is also inactivated (Bartkova et al. 2006; Di Micco et al. 2006). Activation of a DNA damage response by oncogenes therefore presents a strong selection pressure for p53 mutation, potentially explaining the high incidence of p53 mutations in tumours (Gorgoulis et al. 2005; Halazonetis et al. 2008).

1.3.2.2 Limitless replicative potential

Limitless replicative potential can be regarded partly as an evasion of cellular senescence and partly as maintenance of a more 'primitive', undifferentiated cell phenotype. Since these are also properties of stem cells, much has been made recently of the possibility that tumours contain initiating 'cancer stem cells' as well as mutated 'progenitor' cells and peripherally recruited stromal cells, which are not in themselves tumourigenic (Reya et al. 2001; Visvader and Lindeman 2008). These cancer stem cells are thought to be refractive to both chemotherapy and radiotherapy, thus allowing relapse of tumour formation after treatment (Hirschmann-Jax et al. 2004; Setoguchi et al. 2004; Locke et al. 2005; Patrawala et al. 2005; Diehn et al. 2009). Although this appears to contradict the 'clonal expansion' theory of cancer progression, where the single most successful genotype dominates, it is likely that tumour evolution involves a relatively high mutation rate and not all cells would be under selective pressure to maintain stem-like properties (Nowell 1976; Stingl and Caldas 2007). However, recent evidence has suggested that more individual tumour cells may possess 'stem-like' tumourigenic properties than previously thought, as defined by the conditions in which single cells are able to initiate tumours in nude mice (Quintana et al. 2008). In cells which are naturally able to dedifferentiate, such as Schwann cells, the reacquisition of stem-like proliferative ability is relatively simple to achieve, but it is still unclear how 'terminally' differentiated tissues revert to unlimited proliferation, unless the tumour initiates in the stem cell compartment, as increasingly looks to be the case (Stevens and Fields 2002; Perez-Caro et al. 2009).

There are two senescence mechanisms that limit the proliferation of potential cancer cells: replicative senescence due to telomere erosion, and senescence in response to intracellular or extracellular stresses (Mathon and Lloyd 2001). Evading replicative senescence in human cells requires either the activation of telomerase, or an alternative mechanism for lengthening telomeres (ALT) (Meyerson et al. 1997; Neumann and Reddel 2002). Importantly, p53 mutation also allows cells to tolerate telomere shortening, but this additionally results in chromosome fusions and genome instability (Chin et al. 1999). Since activation of oncogenes in itself constitutes an intracellular stress, as described

above, there is already a high selective pressure for p53 mutation in neoplastic cells.

Paradoxically, although senescence is often regarded as a barrier to tumourigenesis, senescence mechanisms may also play a role in promoting cancer (Campisi and Judith 2008). Genotoxic stress, such as that encountered in cells with oncogenic Ras mutations, can lead to a senescent phenotype which involves the secretion of inflammatory cytokines, creating a microenvironment favourable for tumour development (Coppé et al. 2008).

1.3.2.3 Evading apoptosis

As described in section 1.2.2, many oncogenes upregulate signalling through survival pathways, which inhibit pro-apoptotic proteins such as Bad and Bim in cancer cells (Bonni et al. 1999; Goldstein et al. 2009). p53 inactivation prevents the apoptotic response to DNA damage checkpoint activation (Gottlieb and Oren 1998). Both Ras and Myc can promote apoptosis in the absence of cooperating mutations (Fikaris et al. 2006; Murphy et al. 2008). Myc, when activated alone, induces programmed cell death, but when an anti-apoptotic gene such as Bcl-2 is also activated, the combination effectively drives overproliferation (Strasser et al. 1990).

1.3.2.4 Sustained angiogenesis

Angiogenesis allows a tumour mass to grow beyond the maximum size for oxygen diffusion alone to support normal aerobic cellular respiration (Folkman 1992). New blood vessel formation is triggered under hypoxic conditions, but counteracted by p53; hence p53 loss promotes sustained angiogenesis (Teodoro et al. 2006). In addition, cancer cells frequently upregulate glycolytic respiratory pathways in order to better survive in hypoxic conditions (Funes et al. 2007).

1.3.2.5 Loss of anchorage dependence, tissue invasion and metastasis

The invasive and/or metastatic properties of late-stage cancers define them as malignant and are often the cause of fatality, as they grow to the detriment of surrounding healthy tissue (Sahai 2007). Acquisition of an

invasive and metastatic phenotype is associated with altered adhesion to the extracellular matrix, often in response to an inflammatory microenvironment, and changes in signalling to the cytoskeleton to produce a motile cell (Brown and Ruoslahti 2004; Mantovani 2008; Sanz-Moreno et al. 2008; Kim et al. 2009b). For epithelial cancers, the process may involve an epithelial-mesenchymal transition (EMT), characterised by downregulation of the adherens junction protein E-cadherin, a morphological change from a cuboid to a more elongated, 'fibroblast-like' shape due to dismantling of cell-cell junctions and loss of cell polarity, and an increase in migratory characteristics (Thompson et al. 2005). The expression of matrix metalloproteases such as MMP3 can induce EMT, via induction of Rac1b (Radisky et al. 2005). Along with the downregulation of E-cadherin, cells acquire the mesenchymal ability to survive detachment from their neighbours, and no longer undergo anoikis (Derksen et al. 2006).

Anchorage independence forms part of the general self-sufficiency in growth signals that is the first cellular hallmark of cancer. Both the anchorage-controlled kinases, FAK and ILK, have been found to be overexpressed in cancers, allowing signalling independent of integrin ligation (Radeva et al. 1997; Kahana et al. 2002; Hecker et al. 2004; McDonald et al. 2008). Loss of anchorage dependence for survival is an essential requirement for metastasis to distant sites, since it involves movement through blood and/or lymphatic fluid (Mahoney et al. 2002; Geiger and Peeper 2007). Loss of anchorage dependence for proliferation is equally important in metastatic cells, as they must be able to divide in a new tissue microenvironment (Moore et al. 1998; Weber 2008). Anchorage independence in vitro correlates strongly with tumourigenic potential and metastasis in nude mice (Shin et al. 1975; Cifone and Fidler 1980; Nakanishi et al. 2002).

1.4 Genome instability

Genome instability was described by Hanahan and Weinberg as a facilitating mechanism which would help cells acquire the six 'hallmarks of cancer', considering the low probability of accumulating enough genetic lesions to achieve this in a single cell given a normal, stable genome (Hanahan and Weinberg 2000). The concept of genome instability may be defined as an increase in the rate at which DNA and chromosomes are damaged, lost or rearranged, sometimes known as a mutator phenotype (Morgan 2007; Loeb et al. 2008). Although it has been argued that the spontaneous mutation rate is sufficient to explain the number of genetic changes necessary for tumourigenesis (Tomlinson et al. 2002; Bodmer et al. 2008), it is nevertheless clear that the majority of cancers exhibit some form of genome instability, and this often accelerates tumour progression (Lengauer et al. 1998; Cahill et al. 1999; Stoler et al. 1999; Shih et al. 2001; Nowak et al. 2002; Sieber et al. 2003; Kops et al. 2005). Large-scale genomic changes such as translocations and gain or loss of whole chromosomes have long been recognised as a feature of tumour cells – the work of Theodor Boveri led him to propose that aneuploidy is a cause of cancer in 1914 (Boveri 1914), and the Philadelphia chromosome translocation in chronic myelogenous leukaemia was described in 1973 (Rowley 1973). The discovery of oncogenes and tumour suppressors and their roles in cellular transformation dominated cancer research in the latter part of the 20th century (McCormick 1999). Although work initially focused on mitogen signalling pathways, the discovery that inherited cancer predisposition syndromes were caused by mutations in genes involved in DNA damage sensing and repair reignited interest in genome stability as a tumour suppressive mechanism (Kinzler and Vogelstein 1997; Hoeijmakers 2001; Cheok et al. 2005; Deng 2006).

Instability can arise at both the chromosomal and single-nucleotide level, through a multitude of mechanisms (Aguilera and Gómez-González 2008). An increase in point mutation may be due to defects in mismatch repair, such as the MSH2 gene mutated in hereditary non-polyposis colorectal cancer (HNPCC) (Fishel et al. 1993; Papadopoulos and Lindblom 1997). Since short repeating sequences or 'microsatellites' are particularly prone to replication

errors, defects in mismatch repair also lead to microsatellite instability, where the repeating sequences vary in length (Ionov et al. 1993; Thibodeau et al. 1993). Where repeated stretches occur within genes, the variation in number of nucleotides can lead to frame shifts, leading to protein truncations. Truncations of proteins such as the TGFβ receptor, which mediates antiproliferative signals, are extremely common in both HNPCC and sporadic colorectal cancers exhibiting microsatellite instability (Jacob and Praz 2002).

Defects in nucleotide excision repair can also lead to increased point mutation (Hoeijmakers 2001). For example, xeroderma pigmentosum (XP) patients are hypersensitive to UV radiation and have an increased risk of cancer due to mutations in one of the 8 XP genes, encoding damage recognition, helicase and nuclease components of the repair pathway (Cleaver 2005). Defects in DNA repair may initially cause a damage checkpoint response, resulting in a selective pressure for p53 mutation to allow cancer to develop (Spatz et al. 2001).

Chromosomal instability exists in two forms: structural, involving inappropriate chromosome fusions, breakages and translocations, and numerical, where gains or losses of whole chromosomes results in aneuploidy (Gollin 2005). Structural abnormalities may result from:

- DNA double-strand breaks (DSBs). These arise because of defects in DSB repair (Mills et al. 2003), collapsed replication forks due to impaired DNA damage response and replication stress (Halazonetis et al. 2008), or failure to separate or disentangle sister chromatids due to topoisomerase or separase mutations (Shepard et al. 2007; Luo et al. 2009).
- Telomere erosion (‘crisis’) followed by end-to-end fusion of chromosomes (Hastie et al. 1990; DePinho 2000; Hackett et al. 2001).
- Incomplete replication, or rereplication of DNA, leading to breakage, or recombination as intrachromosomal duplications (Blow and Gillespie 2008).

When subjected to replication stress, chromosome breakages, amplifications and deletions occur at common fragile sites due to incomplete replication (Glover et al. 1984; Coquelle et al. 1997). Microsatellite expansion resulting

from mismatch repair defects may also increase breakage at rare fragile sites (Hewett et al. 1998). Fusion of two broken ends may result in the formation of a dicentric chromosome and initiation of a breakage-fusion-bridge cycle, where the chromosomes are attached at two centromeres instead of one, leading to random breakage of the DNA when the chromosome is pulled to opposite poles at anaphase (McClintock 1939). This extreme instability is frequently fatal if it occurs in many chromosomes, even in cells with a defective DNA damage checkpoint (mitotic catastrophe) (Castedo et al. 2004). All these mechanisms involve DNA damage, and so normally activate a pro-apoptotic or pro-senescent response, which acts as a barrier to genome instability (Vaziri et al. 2003; Zhivotovsky and Kroemer 2004; Gorgoulis et al. 2005).

Numerical abnormalities, or aneuploidy, may result from:

- Defects in the spindle checkpoint, such as Bub1 mutation, leading to nondisjunction of chromatid pairs (Cahill et al. 1998; Musio et al. 2003; Weaver et al. 2006)
- Cytokinesis failure, leading to tetraploidy, centrosome reduplication in the following cycle, and increased risk of non-disjunction (Ganem et al. 2007).
- Multipolar spindle formation, due to extra centrosome duplication cycles, the presence of extra centrosomes following cytokinesis failure, or abnormal cell shape, leading to unequal chromatid segregation between more than two spindle poles (Zhou et al. 1998; Meraldi et al. 2002; Sluder and Nordberg 2004; Théry et al. 2007)

Non-disjunction, caused by premature spindle checkpoint inactivation and resulting in trapping of lagging chromosomes at the midbody, will block cytokinesis (Shi and King 2005; Steigemann et al. 2009). Tetraploidy may also occur due to mitotic slippage, where prolonged activation of the spindle checkpoint leads to a return to G1 phase without undergoing mitosis or cytokinesis (Rieder and Maiato 2004; Brito and Rieder 2006). A p53-dependent 'tetraploidy checkpoint' has been reported to act in G1 to eliminate tetraploid cells before progression to S phase (Andreassen et al. 2001; Meraldi et al. 2002; Stukenberg 2004; Fujiwara et al. 2005), but its existence is controversial (Uetake and Sluder 2004; Wong and Stearns 2005; Ganem and Pellman 2007).

It has been argued that aneuploidy most often arises via a tetraploid intermediate, which in turn occurs either due to reduplication of the entire genome, mitotic slippage, or cytokinesis failure (Fujiwara et al. 2005; Storchova and Kuffer 2008). The hypothesis predicts that cells with double the usual number of chromosomes are far more likely to undergo a spindle checkpoint slippage and unequal genome segregation than ordinary diploid cells (Shi and King 2005). The interplay between the various mitotic defects means that aneuploid cell populations often have a highly unstable chromosome number, or will go through a period of rapid variation before settling on a 'favourable' chromosome complement which is then stably passed on to daughter cells (Albertson et al. 2003).

In recent years, the role of aneuploidy in tumourigenesis has resurfaced as a point of intense discussion, with the relative importance of genomic instability as a source of mutation, and the selective pressures acting on tumour evolution, debated (Cahill et al. 1999; Marx 2002; Sieber et al. 2003). The majority view emerging is that aneuploidy can play both permissive and active roles in sporadic cancer development (Pihan 2003; Rajagopalan and Lengauer 2004; Weaver et al. 2007; Chandhok and Pellman 2009). Colon cancers, other than those initiated due to defects in mismatch repair, tend to show high levels of chromosomal instability, but not microsatellite instability (Lengauer et al. 1997). This dichotomy lends support to the view that aneuploidy is not simply a side effect of tumour development: chromosomal abnormalities do not occur where another form of genomic instability exists (Vogelstein, in (Marx 2002)). The discovery of a subset of tumours that are both stably diploid and free of hypermutation was a puzzle until it was found that cancers may also have altered epigenetic regulation (Hawkins et al. 2001; Jones and Baylin 2007). Genes can therefore be activated or silenced without showing up as mutations on comparative genomic arrays, with cancers in some cases giving single nucleotide polymorphism (SNP) patterns almost indistinguishable from normal cells. Despite being genetically stable, these tumours can be aggressive, indicating that although genome instability is a contributing factor in many cancers, it is not necessary for malignancy (McKenna et al. 2008).

1.5 Introduction to this thesis

As described in the previous sections, many aspects of cell cycle regulation and its relevance in cancer have already been elucidated. Anchorage independence has been identified as a key property of cancer cells, and is thought to promote metastasis (Cifone and Fidler 1980; Wang 2004; Weber 2008). However, the genetic changes known to correlate with anchorage independence in cancer cells do not explain some of the observations made in anchorage dependent models of cell transformation, as described below. The aim of this work was therefore to investigate the mechanisms underlying the establishment of anchorage dependence in tumourigenesis, using a genetically defined stepwise transformation model.

The model I employed made use of the well-characterised cooperation between the viral SV40 Large T antigen (LT) and the oncogenic Ras G12V mutant (Michalovitz et al. 1987; Clark et al. 1988; Hirakawa and Ruley 1988; Ridley et al. 1988). LT is an oncoprotein from the tumour virus SV40, which transformed cells in culture (Todaro et al. 1964). LT was subsequently found to bind and inhibit p53, Rb, and the related pocket proteins p107 and p130, some of which were discovered as LT-binding proteins (Lane and Crawford 1979; DeCaprio et al. 1988; Dyson et al. 1989; Hannon et al. 1993). Both the p53- and Rb-binding activities of LT were found to cooperate with oncogenic Ras in inducing full transformation of primary cells (Beachy et al. 2002). Lesions in p53, Rb and Ras are extremely common in many types of cancer, and these changes have been successfully modelled in primary human cells expressing telomerase (Hahn et al. 1999; Elenbaas et al. 2001). Here I have used primary rat Schwann cells, which do not require exogenous telomerase for immortalisation (Mathon et al. 2001), and expressed LT and oncogenic Ras in a stepwise manner, to investigate their effects on anchorage dependent proliferation.

Previous work in the lab (Mitchell et al. 2003) has established that both normal primary Schwann cells, and cells expressing LT, do not form colonies in soft agar and are therefore anchorage dependent. Additional expression of oncogenic Ras^{G12V} in LT-expressing cells confers anchorage independence, and both p53 and Rb inactivation by LT, and Ras activation, are required to achieve

this – any combination of two is not sufficient. Loss of p16(INK4A) was shown to substitute for Rb inactivation in allowing proliferation in suspension, as it effectively removes the same Rb-dependent checkpoint, but again this was not sufficient to induce anchorage independence in combination with p53 inactivation alone (Mitchell et al. 2003). At the time of beginning work on this project, only oncogenic Ras was known to complement the effects of LT and allow anchorage-independent proliferation as measured by colony formation in soft agar suspension. The anchorage dependence of LT-expressing cells is puzzling, since previous reports would suggest that Rb inactivation would remove the anchorage requirement for cell-cycle progression (see section 1.2.3).

Presumably because of the fundamental importance of correct cell cycle regulation, mammalian cells have developed secondary, 'cryptic' checkpoint mechanisms that may not be apparent during normal cell division, but are revealed when other tumour suppressive mechanisms are lost. This 'belt and braces' approach can be exploited when treating cancer: by either strengthening the secondary checkpoint, targeting pathways that are redundant in normal cells, or thwarting the cancer cell's checkpoint evasion mechanism, we can hope to frustrate cancerous proliferation with minimal disruption to normal cell division. For example, cancer cells with multiple centrosomes may evade potentially catastrophic multipolar divisions by grouping the extra centrosomes together to create a normal bipolar spindle. If this mechanism is disrupted, by removing the non-essential kinesin motor protein HSET, cells with multiple centrosomes are selectively killed (Kwon et al. 2008). A similar concept has already been used in Phase I trials in the clinic, to inhibit the non-essential poly(ADP-ribose) polymerase PARP in BRCA2-deficient cancers, which are especially sensitive to DNA single-strand breaks (Ashworth 2008; Plummer et al. 2008). The cancers are thought to be unusually dependent on the base-excision repair pathway mediated by PARP in the absence of the BRCA2 homologous recombination pathway (Bryant et al. 2005; Farmer et al. 2005). Here, we hypothesised that LT-expressing cells use a secondary 'anchorage checkpoint' mechanism, in the absence of the usual Rb-dependent checkpoint that senses loss of cell attachment. This work aimed to clarify the mechanism of this novel anchorage checkpoint in cells lacking p53 and Rb function,

focusing on the molecular basis of cell cycle arrest in suspension, as this could represent a critical barrier to transformation and potential means of suppressing tumourigenesis in checkpoint-deficient cells.

Chapter Two –Materials and Methods

2.1 Cell Culture

2.1.1 Schwann cell culture

Primary Schwann cells purified from the sciatic nerves of 7-day-old rats (see Cheng et al. 1995) were maintained in culture on plastic dishes [Nunc] coated with poly-L-lysine [Sigma P-6282]. Cells were routinely cultured in DMEM [Gibco 11880, 1mg/ml D-glucose] supplemented with 3% stripped foetal calf serum (FCS) [Biosera], 4mM L-Glutamine [Gibco 25030], 0.1mg/ml kanamycin [Sigma K1377], 2µg/ml gentamicin [Gibco 15710], 1µg/ml forskolin [Calbiochem 344270], and glial growth factor (GGF), produced in the lab. Cells were maintained in the above medium, incubated at 37°C in 10% CO_2 and 95% humidity. Medium was changed every two days, and cells were passaged every three days (seeding $7x10^5$ LT-expressing cells or $1.3x10^6$ normal Schwann cells per 15cm diameter dish), on reaching approximately 80% confluency.

2.1.2 Phoenix cell culture

The retroviral packaging cell line, Phoenix [Nolan lab, Stanford University], was used to introduce genetic material into Schwann cells, as detailed below. Phoenix cells were routinely cultured on uncoated plastic dishes, in DMEM+Glutamax™ [Gibco 31966, 4.5mg/ml D-glucose] supplemented with 10% foetal bovine serum (FBS) [Sigma], 0.1mg/ml kanamycin [Sigma K1377], and 2µg/ml gentamicin [Gibco 15710]. Before seeding, cells were passed through an 18G needle, to ensure an even distribution on the plate. Cells were incubated at 37°C in 10% CO_2 and 95% humidity.

2.1.3 MEF cell culture

Mouse embryo fibroblasts (MEFs) from wild-type and p27Δ51 knock-in mice (Kiyokawa et al. 1996) were a kind gift from Dr Andrew Koff (Memorial Sloan Kettering Cancer Center, New York, NY) and were cultured in DMEM+Glutamax™ [Gibco 31966] supplemented with 10% FBS [Sigma],

plus antibiotics as above. Cells were incubated at 37°C in 7% CO2, 95% humidity and 3% O2 conditions.

2.1.4 Generation of cells by Phoenix infection

Protocol for Phoenix transfection and retroviral infection of cells

1) 5 million Phoenix cells were seeded on a 10cm plate for each transfection and left to settle overnight.
2) 5µg of plasmid DNA was mixed with 500µl of serum free medium (DMEM 31966 + antibiotic only), followed by 17.5µl of PLUS™ reagent [Invitrogen 18324-012], and left at room temperature for 15 minutes. A separate tube was prepared for each of the constructs and for the control construct pBIRD-GFP.
3) In fresh tubes, 25µl of Lipofectamine™ reagent [Invitrogen 11514-015] was mixed with 500µl of serum free medium for each transfection, and the DNA/PLUS™ mix from step 2 added. The transfection mixture was left for a further 15 minutes at room temperature to allow DNA/lipid complexes to form.
4) Medium on Phoenix cells was replaced by 4ml serum-free medium per plate, washing once with serum-free medium.
5) The DNA/lipid complexes from step 3 were carefully added dropwise to the Phoenix plates, rocked gently to mix, and incubated under usual culture conditions for 3-4 hours to deliver the DNA to cells.
6) Transfection medium was removed and replaced by normal Phoenix medium containing 10% serum. Medium was replaced after 24 hours with 6ml fresh medium and the cells were left overnight to produce virus.
7) 4ml viral supernatant was collected from each plate, Polybrene® [hexadimethrine bromide, Sigma H9268] was added at a final concentration of 8µg/ml, and the solution was filtered to remove cell debris before adding to subconfluent Schwann cells/MEFs neat. Phoenix plates were topped up with an extra 4ml medium to continue virus production.

8) Schwann cells/MEFs were incubated for 3 hours with the viral supernatant, then left to recover for 2 hours in normal medium. A second, 4 hour, infection was then carried out as in step 7, followed by recovery overnight. The following day, cells were subjected to a third round of infection for 3 hours, before final recovery in normal medium.
9) Infected cells were left to recover for two days in normal medium, then cultured for at least 2 weeks in medium containing G418 selection antibiotic [Calbiochem 345812] at 1.25mg/ml, to remove non-vector expressing cells and ensure stable vector expression.

Schwann cells "NSLT" and "NSLTRas"

Phoenix producer cells were transfected with either LXSN empty vector, or H-Ras^{G12V} LXSN, according to the protocol above. Normal Schwann cells (NS) stably expressing SV40 LT, from the pBabe-Puro vector, had been previously produced in the lab. These cells were infected at passage 6 with LXSN or H-Ras^{G12V} LXSN retroviral supernatant from the Phoenix cells, to create the non-clonal "NSLT" and "NSLTRas" cell populations used in this thesis. Control infections using pBIRD-GFP-carrying retrovirus were carried out in parallel to estimate the rate of infection. Rates of infection were consistently above 50% using this protocol.

shRNA-expressing NSLT cells "2", "3", "7", "itoh", and "control"

Phoenix producer cells were transfected with the RNAi-Ready pSIREN-RetroQ-ZsGreen vector [Clontech 632455] carrying each of the above 5 inserts (see section 2.4.2). NSLT cells were infected with retroviral supernatant produced following Phoenix cell transfection according to the protocol above.

WT and p27Δ51 MEFs expressing LT

Phoenix producer cells were transfected with either the Babe-Puro empty vector, or LT-Babe-Puro, using the protocol above. WT and p27Δ51 MEFs were infected with retroviral supernatant as below, except that the second and third rounds of infection were omitted and cells were used in experiments immediately following overnight recovery, with no selection. Control

infections using pBIRD-GFP-carrying retrovirus were carried out in parallel to estimate the rate of infection.

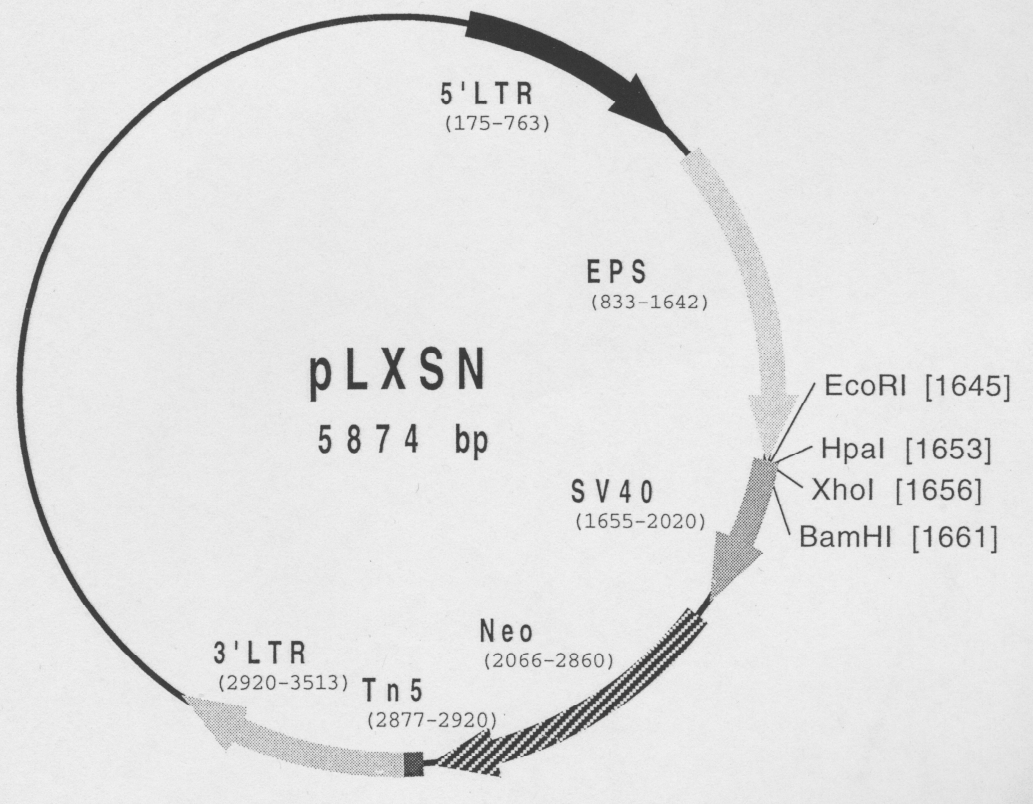


Figure 2- 1: pLXSN.

The pLXSN retroviral vector (Miller and Rosman 1989) was used as an empty-vector control to generate "NSLT" cells, used in most experiments in this thesis. The H-Ras^{G12V}LXSN vector has the H-Ras^{G12V} oncogene sequence cloned into the EcoRI site and was used to generate "NSLTRas" cells in parallel experiments. The "SV40" sequence contains an early promoter. The retroviral long terminal repeat (LTR) sequence drives strong expression of the cDNA. The "Neo" gene confers resistance to the selection antibiotic G418.

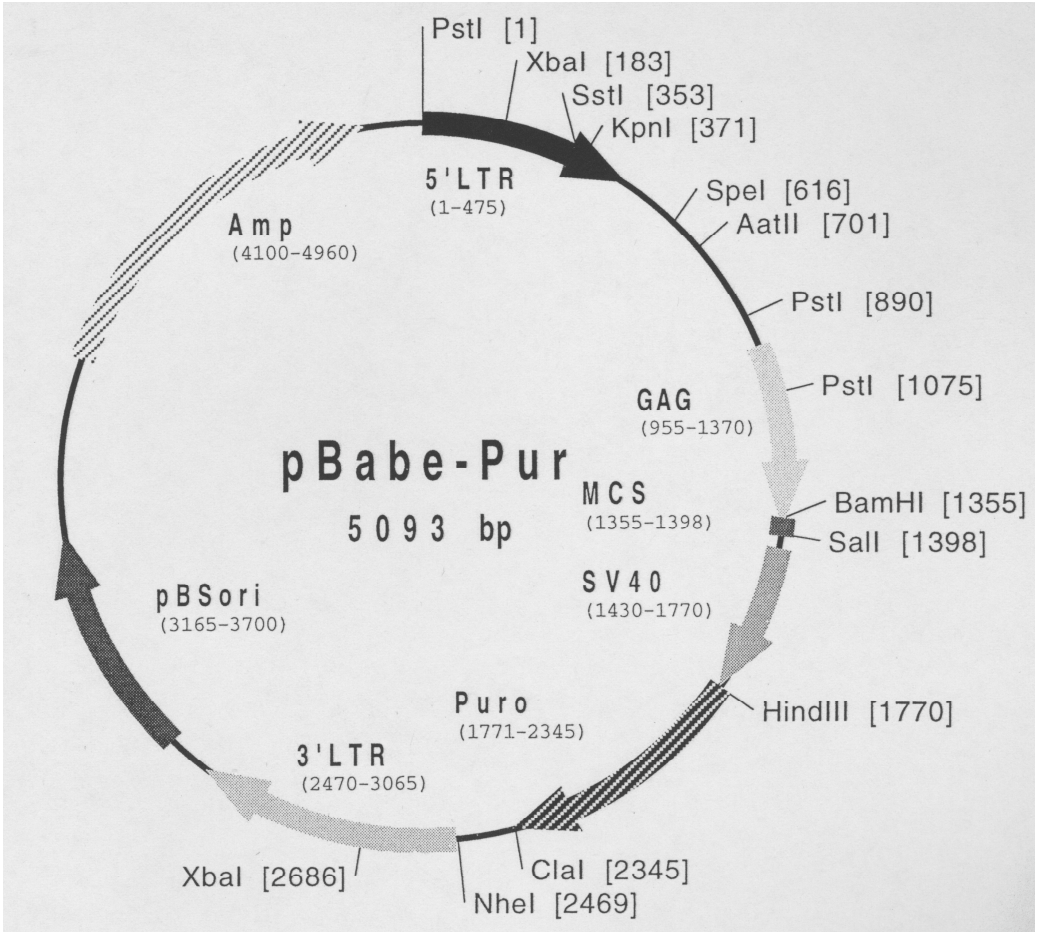


Figure 2- 2: pBabe-Puro.

pBabe-Puro retroviral vector was used both as an empty-vector control, and with SV40 LT cDNA inserted at the multiple cloning site (MCS) shown above, for MEF infections and expression of LT in Schwann cells. The "SV40" sequence contains an early promoter. The retroviral long terminal repeat (LTR) sequence drives strong expression of the cDNA. The "Puro" gene confers resistance to the selection antibiotic Puromycin.

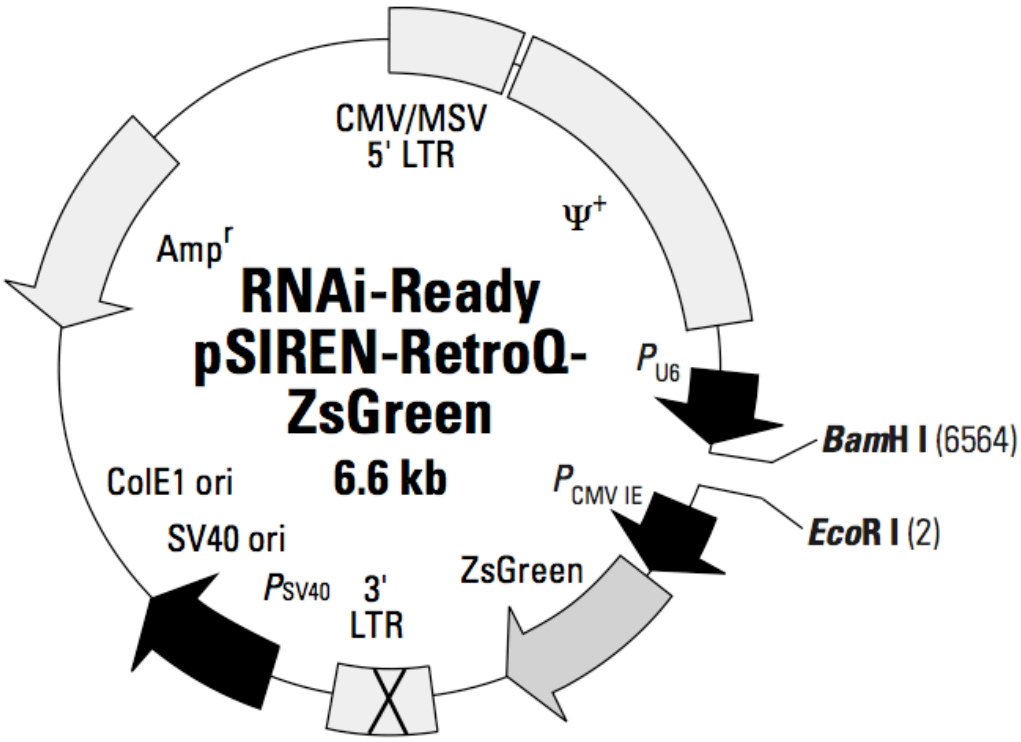


Figure 2- 3: pSIREN-RetroQ-ZsGreen.

The RNAi-Ready pSIREN-RetroQ-ZsGreen vector [Clontech 632455] was used for stable expression of short hairpin RNA (shRNA) constructs in Schwann cells (see section 2.4.2). shRNA duplexes were cloned into the vector between the BamHI and EcoRI sites, where insert expression is driven by the U6 promoter. The CMV promoter drives constitutive expression of the *Zoanthus sp.* green fluorescent protein ZsGreen, allowing enrichment of the vector-expressing population by FACS. Vector map reproduced from Clontech vector information PT3777-5, freely available online.

2.1.5 Suspension culture and cell retrieval

Schwann cells (NS, NSLT and NSLTRas) were seeded in centrifuge tubes, in medium as described in section 2.1.1 but also containing 1.8% dissolved methylcellulose [Sigma M0512], to form a semisolid hydrogel suspension.

To make 1.8% methylcellulose medium:

1) A 500ml Duran bottle containing a magnetic stir bar and 9g methylcellulose powder [Sigma M0512] was autoclaved.
2) A 500ml bottle of DMEM [Invitrogen] was warmed to 37°C and, under a laminar flow cabinet, poured bit by bit onto the autoclaved methylcellulose, stirring all the time with a sterile pipette to disperse clumps. The bottle was then mixed on a heated magnetic stirrer for 30 minutes-2 hours, to make sure the powder was dispersed evenly into a cloudy solution.
3) The bottle of semi-dissolved methylcellulose was next placed in a bucket of ice overnight to dissolve completely.
4) Methylcellulose medium was kept at 4°C and poured out to use as needed. Serum, antibiotic etc. were added just before use, as for normal culture medium.

To seed cells in suspension,

1) The required volume of methylcellulose medium was warmed to 37°C, serum and factors were added as in section 2.1.1, and the gel poured into tubes as needed. 10ml methylcellulose suspension in a 50ml centrifuge tube was used for cell counts, and scaled up to 50ml suspension in a 250ml tube for larger samples e.g. for Western blotting.
2) Cells were trypsinised and resuspended in a small volume of medium (1-5ml depending on size of tube) and squirted into the centre of the gel using a sterile pipette under the surface of the methylcellulose. 800,000 cells were seeded in 10ml, or 4 million in 50ml suspension.
3) The lid was closed and the tube slowly inverted at least 20 times to ensure cells were evenly mixed. Tubes were kept upright in the

incubator in a rack or beaker, with lids loosely attached to allow equilibration with the CO_2-controlled atmosphere.

4) Suspension cultures were maintained in this way for a maximum of 72 hours, as there was no way of replacing the medium without disturbing the cells.

To retrieve cells:

1) Methylcellulose suspension was diluted first 1:2 with warm DMEM + 1% serum, mixing thoroughly, then the tube was filled with further, cold, DMEM + 1% serum (to 50 or 250ml), inverted to mix, and the tubes placed on ice.
2) Tubes were centrifuged [Beckman Coulter J6-M] at 500 *g* for 10 minutes at 4°C.
3) Dilute medium was aspirated to the 5ml mark. Usually there was no compact pellet at this stage, and cells were spread around the sides of the conical base of the tube.
4) Cells were resuspended in 10ml ice-cold PBS, carefully dislodging them from the walls of the tube, and transferred to a clean 15ml tube.
5) The suspension was centrifuged again at 500 *g* for 5 minutes at 4°C. After this, a more compact pellet usually appeared.
6) (a) For counting, supernatant was aspirated down to the pellet, and 500µl trypsin was added to dissociate cells. The suspension was pipetted up and down either very gently once or twice using a 1ml Gilson tip (for anchorage-dependent cells), or 5-10 times fairly vigorously using an 18G needle and syringe (for colony-forming cells). 4.5ml normal medium containing serum was added to inactivate the trypsin, and the resulting suspension was counted in isotonic solution [Beckman Coulter Isoton® II diluent] using a Coulter counter. Only one tube was processed in trypsin at a time.
6) (b) For replating, supernatant was aspirated and the pellet resuspended gently in warm medium, and the cells plated straight away.
6) (c) For freezing a pellet for lysis, supernatant was aspirated thoroughly and the pellet was re-washed in 1ml ice-cold PBS to remove residual

methylcellulose. The washed cells were transferred to a 1.5ml tube and centrifuged for 3 minutes at 3500 *g* in a cooled minifuge at 4°C. The pellet was then snap frozen in liquid nitrogen. (N.B. For cyclin IP or kinase assay, pellets were only used 'fresh' and lysed directly without snap freezing.)

2.1.6 Harvesting attached cell pellets

Medium was removed from cells to be harvested and the dishes immediately placed on ice. Cells were washed twice in ice-cold phosphate-buffered saline (PBS), and excess PBS removed. Each dish was tilted and the cells scraped quickly downwards using a clean rubber policeman. The collected cells were then taken up using a 1ml pipette and transferred to a 1.5ml tube placed on ice. 500µl extra ice-cold PBS was used to wash off any remaining cells and transferred to the same tube. Cell samples were centrifuged at 3500 *g* in a cooled minifuge at 4°C for 3 minutes, all supernatant was removed and pellets were snap frozen in liquid nitrogen. (N.B. For cyclin IP or kinase assay, pellets were only used 'fresh' and lysed directly without snap freezing.)

2.1.7 Flow cytometry

BrdU was added to medium or methylcellulose suspension 1 hour before cell harvesting, and mixed thoroughly. Samples to which BrdU had not been added were processed in parallel with the test samples for all conditions. Upon retrieval, the cell pellets were washed in ice-cold PBS + 1mM EDTA, resuspended in 200µl of the same and then fixed in 2ml 80% ice-cold ethanol while vortexing. Ethanol-fixed samples were kept at 4°C for up to two weeks until needed. Samples were then washed in ice-cold PBS + 1mM EDTA to remove ethanol, and treated with 2M HCl for 30 minutes at room temperature (RT) to denature DNA, followed by 2 washes in PBS + 1mM EDTA and one in PBS-T (PBS + 0.1% BSA + 0.2% Tween 20). The washed cell pellet was incubated for 20 minutes with 2µl anti-BrdU antibody [Roche 11170376001, clone 9318] at room temperature in the dark. After two more washes with PBS-T, the pellet was incubated with 50µl AlexaFluor® 488 fluorescent secondary antibody [Molecular Probes] at 1:250 in 0.1% BSA/PBS for 20 minutes at room

temperature in the dark. The stained cells were then washed once more in PBS + 1mM EDTA and resuspended in 300µl propidium iodide (PI) solution (50µg/ml PI [Sigma P-4864], 100µg/ml RNase A [Qiagen], 0.1% BSA [Sigma], in PBS + 1mM EDTA). BrdU- and PI-stained cells were analysed the following day using a FACSCalibur flow cytometer [Becton Dickinson] and Cellquest Pro software. 10,000 events were collected for each sample, gated either to include only single cells with a DNA content between 2N and 4N (Chapter Three), or all single cells with >2N DNA content (see Figure 5-1).

2.1.8 Roscovitine treatment

For FACS analysis

Attached cells were treated with the CDK inhibitor Roscovitine [Calbiochem 557360] added to the medium as a 10mM stock in DMSO. Final concentrations of drug ranged from 5-50µM, and the treatment was left on for 24 hours. For the last hour of treatment, BrdU was added to the medium, before harvesting, fixing and staining cells as above.

For analysis of giant nuclei

Cells were cultured on PLL-coated glass coverslips for 3 days, with Roscovitine added to the medium as above, and replenished every 24 hours. After 72 hours' treatment, cells were fixed for 20 minutes in 4% formaldehyde/PBS at room temperature, washed twice in PBS, and incubated with Hoechst [Bisbenzimide H 33342, Fluka 14533] at a final concentration of 170ng/ml in 0.1% BSA/PBS for 1 hour. Coverslips were then washed 6 times by dipping sequentially into small containers of PBS, and mounted on glass microscope slides using Prolong Gold [Invitrogen P36930]. Slides were examined using a Zeiss Axioplan 2 fluorescence microscope. Using hand-held counters to keep tally, 100 nuclei were counted using the Hoechst channel, and the number of "giant" nuclei – that is, with an area approximately twice that of the majority of nuclei in the field, was noted.

2.1.9 Soft agar colony formation assays

For standard Schwann cell and MEF assays:

Cells were seeded in 6-well plates at 4000 cells per well, in a top layer of 0.6% agarose [Seaplaque®, Lonza 50101] over a bottom layer of 0.8% agarose in 5:6 normal MEF or Schwann cell medium: PBS. PBS was added initially to the agarose powder and autoclaved, followed by dilution of the warm melted agarose in medium. MEF colonies were counted after 1 week in 10 fields per well, using a 4x objective phase contrast microscope. Schwann cell colonies were stained with MTT [Calbiochem 475989] after 2 weeks, by adding 100µl of a 10mg/ml solution to each well and incubating overnight.

To test for rare oncogenic transformation in Schwann cells (see section 5.6):

Cells from either attached or replated (2 passages on normal dishes following 72 hours in methylcellulose suspension) cultures were used. 500 000 cells per 15cm plate were seeded in a layer of 0.5% agarose on top of a bottom layer of 1% agarose [Seaplaque®, Lonza 50101] dissolved in normal medium. The following day, the plates were overlaid with 5ml fresh culture medium and fed with another 5ml each week. Colonies were counted after 4 weeks, in 30 fields per plate, using a 4x objective phase contrast microscope [Olympus].

Soft agar for time-lapse:

4000 NSLT cells per well of a 12-well plate were seeded in 500µl 0.6% agarose. When set, the thin layer of agar was overlaid with fresh medium and the plate humidified by filling unused wells with sterile water. The plate was incubated in a 37°C chamber under a phase contrast microscope and photographs of individual cells were taken every 15 minutes.

2.1.10 Use of kinase inhibitors on NSLTRas suspended cells

Following NSLTRas cell seeding into methylcellulose, the MEK inhibitor U0126 [Promega V1121] or the PI3K inhibitor LY294002 [BioMol ST-420] were added to the suspension at 20µM final concentration in DMSO. Tubes were inverted slowly 20 times to mix cells with inhibitor. DMSO was added at 1:1000 as a vehicle control. Cells were then retrieved and counted in duplicate shortly after seeding, and a day later.

2.2 Protein Analysis

2.2.1 Western blotting

Harvested cell pellets were lysed in RIPA buffer (1% Triton X100, 0.5% sodium deoxycholate, 50mM Tris pH 7.5, 100mM NaCl, 1mM EGTA pH 8, 20mM NaF, 100µg/ml PMSF, 14µg/ml aprotinin, 1mM sodium orthovanadate). Samples were vortexed and incubated on ice for 15 minutes, vortexing every 5 minutes. Lysates were then centrifuged at 12000 *g* in a cooled minifuge at 4ºC for 15 minutes before transferring the cleared supernatant to a fresh 1.5ml tube. Protein concentration was measured against standard BSA solutions using the BCA microplate assay [Pierce] and extra RIPA added as needed to equalise protein concentration in all samples. 4x Laemmli sample buffer (200mM Tris-HCl pH 6.8, 8%(w/v) SDS, 40% glycerol, 0.4% (w/v) bromophenol blue, 400mM DTT) was then added and samples boiled at 95°C for 5 minutes to denature protein. Samples were loaded onto polyacrylamide gels (see below), resolved by SDS gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes [Millipore Immobilon-P]. Membranes were blocked for 1hr at RT in 5% milk powder/TBST (Tris-buffered saline + 0.05% Tween 20), or according to the antibody directions. Blocked membranes were incubated overnight in primary antibody diluted in block solution, with rolling agitation, before washing in TBST and incubating for one hour in horseradish peroxidase-conjugated secondary antibody diluted in block. Membranes were then washed 4 times in TBST and once in TBS before chemiluminescent detection using ECL Plus™ reagent [GE Healthcare].

<u>Recipe for discontinuous polyacrylamide gels</u>:

Resolving gel: Acrylamide/Bis 30%/0.8% solution to required final polyacrylamide concentration (5-15%)
373mM Tris pH 8.8
0.1% sodium dodecyl sulphate (SDS)
0.04% tetramethylethylenediamine (TEMED)
0.04% ammonium persulphate (APS)

Stacking gel: 5% acrylamide/bis

125mM Tris pH 6.8

0.5% SDS

0.12% TEMED

0.06% APS

Antibodies used for Western:

Protein	**Antibody**	**Dilution**	**Species**
Cyclin D1	sc-450	1:5000	Mouse
Cyclin E	sc-481	1:200	Rabbit
Cyclin A	sc-596	1:5000	Rabbit
Cyclin B1	sc-595	1:500	Rabbit
CDK4	sc-260	1:1000	Rabbit
CDK6	Neomarkers Ab-3	1:1000	Mouse
CDK2	sc-163	1:2000	Rabbit
CDK1	sc-747	1:500	Rabbit
p21	sc-397-G	1:500	Goat
p27	sc-1641	1:500	Mouse
ß-tubulin	Sigma T4026	1:10,000	Mouse

All antibodies are from Santa Cruz Biotechnology if not otherwise stated. Secondary antibody dilutions were 1:2000, except for ß-tubulin, for which a 1:5000 dilution was used. For immunoblotting following immunoprecipitation (IP)(see below), double the stated antibody concentration was used.

2.2.2 Antibody-sepharose cross-linking

Cyclin A antibody [Cancer Research UK clone E72] was stably cross-linked to protein G-sepharose beads [Sigma P3296] in order to avoid interference of the precipitating antibody heavy and light immunoglobulin chains with the pulled-down proteins visible by Western blot.

1) 1mg antibody was diluted to 5ml in Borate buffer (200mM sodium tetraborate decahydrate, pH 9.0) and added to 500µl of packed beads.
2) The mixture was rotated at RT for 1 hour to bind antibody to sepharose.

3) Beads were pelleted (5 minutes at 50 *g* in bench-top centrifuge), then washed for 2 minutes rotating in Borate buffer, pelleted and washed again.
4) Beads were pelleted once more and diluted to 5ml in Borate buffer containing 20mM dimethyl pimelimidate (DMP) cross-linking agent.
5) The mixture was rotated at RT for 30 minutes to cross-link antibody to beads.
6) Beads were pelleted and resuspended in 5ml Ethanolamine buffer (200mM ethanolamine, pH 8.0), rotating at RT for 5 minutes to stop the cross-linking reaction.
7) Beads were pelleted and resuspended in 5ml fresh Ethanolamine buffer, rotating for 1 hour at RT to quench unreacted DMP.
8) Beads were pelleted and washed in 5ml NP40 buffer (1% Nonidet P40, 150mM NaCl, 50mM Tris pH8, 20mM NaF, 100µg/ml PMSF, 14µg/ml aprotinin, 1mM sodium orthovanadate, 10mM sodium pyrophosphate), and kept as a 50:50 bead: buffer slurry for experiments.

2.2.3 Immunoprecipitation and kinase assays

Freshly harvested, unfrozen cell pellets were used to help preserve protein complex integrity. Pellets were lysed in NP40 buffer (see above) and the protein concentration measured using the BCA assay as for Western blotting. 1000µg protein was used for immunoprecipitation (IP) followed by Western blot; for kinase assays, 100µg protein was used for cyclin B IPs, 150µg protein for cyclin A IPs, and 300µg protein for cyclin E IPs. Lysates were made up to 1ml in NP40 buffer and pre-cleared with either protein A- or protein G-sepharose beads [Sigma P9424 or P3296] rotating for 2 hours at 4°C, before incubating for 1 hour with either:

- cyclin E antibody (10µl Santa Cruz sc481) for 1 hour followed by the addition of 50µl protein A bead slurry for 1 hour;
- cyclin B antibody (3µl Santa Cruz sc245) for 1 hour followed by the addition of 50µl protein G bead slurry for 1 hour;
- protein G beads cross-linked to cyclin A antibody [Cancer Research UK clone E72], for 1 hour.

Beads were washed 3 times in ice-cold NP40 buffer and either boiled in 50µl 1x Laemmli buffer before using for Western blot as above, or further washed twice in ice-cold kinase assay buffer (50mM Tris pH 7.5, 10mM MgCl2, 1mM DTT) plus 1mg/ml BSA for kinase assay. Kinase assay samples were incubated with γ-^{32}P ATP [Amersham AA068-250µCi] at 1:100 in kinase assay buffer plus 200µg/ml purified histone H1 substrate [Sigma] at 37°C for 30 minutes. Radioactive samples were run on polyacrylamide gels as for Western blot, and the gels were then fixed for 10 minutes in 40% methanol + 10% acetic acid, vacuum dried and exposed to film or phosphor screen. Screens were scanned and densitometry was used to determine the intensity of the bands. To compare between experiments using slightly different levels of radioactivity (due to the half-life of the γ-^{32}P ATP), the intensity of each band was quantified relative to the NSLT attached sample.

2.2.4 p27 immunodepletion

250µg of protein lysate from NSLT attached and suspended cells was either pre-cleared using 50µl protein A-sepharose beads, or immunodepleted using 20µl p27 antibody [Santa Cruz sc-528] together with beads, rotating at 4ºC for 1 hour. Beads were pelleted and discarded, while the cleared/depleted supernatant was subjected to 2 more rounds of clearing or depletion, respectively. Samples of lysate were taken before and after each depletion to monitor the decreasing p27 levels by Western blot. The final control (non-specifically cleared) and p27-depleted (lacking all p27-bound protein) samples from both attached and suspended cell lysates were then immunoprecipitated with 50µl cyclin A-crosslinked beads (see section 2.2.2), and the immunoprecipitate blotted for CDK2 [Santa Cruz sc-163, 1:1000].

2.3 Microscopy

2.3.1 Immunofluorescence

BrdU immunofluorescence

Cells were seeded the day before staining, on 13mm diameter poly-L-lysine coated glass coverslips in 4-well plates at densities ranging from 15,000

to 30,000 cells/well. BrdU was added for 4 hours before fixing the cells with 4% formaldehyde in PBS for 20 minutes at room temperature. If assaying suspended cells, BrdU was added to the methylcellulose and mixed thoroughly by inversion before incubating for 4 hours, retrieving the cells as normal and seeding them on coverslips as above. Previously suspended cells were allowed to attach to the coverslips (30mins-1hr) before fixing. Fixed cells were then washed twice in PBS and permeabilised for 30 minutes at room temperature (RT) in 0.5% TritonX100 + 2M HCl to denature DNA. After 2 more PBS washes, cells were blocked for 15 minutes at RT in 3%BSA/PBS. Blocked coverslips were incubated with primary antibody to BrdU [Roche clone BMC 9318] at 1:300 in 0.1% BSA/PBS, for 1 hour at RT. Coverslips were then washed 6 times by dipping sequentially into containers of PBS, and incubated with the secondary antibody, AlexaFluor® 488 anti-mouse [Invitrogen] diluted at 1:300 in 0.1% BSA/PBS, plus 1:6000 Hoechst. Following antibody staining, coverslips were washed again 6 times in PBS, finally rinsing in water before mounting inverted on glass slides with ProLong Gold [Invitrogen]. BrdU staining was viewed using an Axioplan 2 fluorescence microscope [Zeiss].

LT immunofluorescence

20,000 cells were seeded per coverslip. The following day, cells were fixed in 4% formaldehyde/PBS for 15 minutes at RT and washed twice in PBS. Cells were then permeabilised in 0.5% TritonX100/PBS for 15 minutes at RT, before blocking for 1 hour at RT in DMEM/10% FCS. Coverslips were washed in PBS and incubated for 1 hour at RT in the dark with primary antibodies to SV40 [Fitzgerald Industries International, pAb419] at a 1:50 dilution in 3% BSA/PBS. After washing 6 times in PBS, coverslips were again incubated for 1 hour at RT in the dark, with secondary antibodies (FITC-conjugated anti-mouse 1:300) diluted in 3% BSA/PBS + 1:5000 dilution Hoechst. Coverslips were finally washed 6 times in PBS and once in water before mounting on glass microscope slides in ProLong Gold [Invitrogen].

2.3.2 Hoechst/ CellTracker

Cells were either grown on coverslips overnight at approximately 20,000 cells per well, or cultured in methylcellulose for 24 hours, then retrieved, washed and allowed to settle onto coverslips. CellTracker Red stock was added to the medium at 5μM final concentration and the cells incubated at 37ºC for 30 minutes before washing once with medium, replacing with fresh medium and incubating for a further 30 minutes. CellTracker-stained cells were then fixed for 20 minutes in fresh 4% formaldehyde/PBS at RT, washed twice in PBS, and incubated with Hoechst at 1:6000 in 0.1% BSA/PBS for 1 hour. Coverslips were then washed 6 times by dipping sequentially into small containers of PBS, and mounted on glass microscope slides using ProLong Gold [Invitrogen]. Slides were examined using a Zeiss Axioplan 2 fluorescence microscope as before, and two counts were made using hand-held counters: (1) Count 100 whole cells using the CellTracker channel, and note how many are binucleate by switching to the Hoechst channel; (2) Count 100 nuclei using the Hoechst channel, and note how many appear "giant" – that is, have an area approximately twice that of the majority of nuclei in the field. Blind labelling of slides was used to eliminate bias between samples.

2.3.3 Metaphase spreads

1) Cells were seeded on 10cm dishes, either passaging normally for attached cells, or immediately after retrieval from methylcellulose suspension.
2) After 48 hours, or directly after plating for cells retrieved from methylcellulose suspension, demecolcine was added (Sigma D1925, final conc. 0.05μg/ml) to the medium to block cells at metaphase.
3) After 2 hours in demecolcine, cells were trypsinised into tubes and centrifuged for 10 minutes at 30 *g* (the low speed is essential to keep cells intact).
4) Supernatant was removed. Cells were gently resuspended in 2ml warm 0.075M KCl solution.
5) Tubes were incubated at room temperature for 12 minutes to swell the cells.

6) Tubes were centrifuged for exactly 5 minutes at 30 *g*, in order to pellet swollen cells without bursting them.
7) Supernatant was removed. 3ml fresh, ice-cold Carnoy's fixative (3:1 dried methanol: glacial acetic acid) was gently added down the side of the tube, pipetting gently but rapidly to prevent cells from clumping.
8) Tubes were left to fix at room temperature for 30 minutes.
9) Tubes were centrifuged again for 5 minutes at 30 *g*. Supernatant was removed and cells resuspended in fresh fixative.
10) Steps 8 and 9 were repeated, finally resuspending cells in 300µl fixative.
11) 20µl cell suspension was dropped onto ultra clean microscope slides from a height of 40cm. Slides were held vertically at an angle so that the drop spread out rapidly upon impact, and left propped at an angle to dry.
12) Slides were washed in 70% acetic acid and left to dry.
13) Vectashield mounting medium with DAPI was used to mount spreads under large square coverslips.
14) Chromosomes were observed under a fluorescence microscope [Axioplan 2, Zeiss].

Several metaphase spreads were photographed for each sample and then the chromosomes counted using the CellCounter application for ImageJ. Spreads with well-separated chromosomes were preferentially chosen, but some had one or two overlapping, which meant that counts were only accurate to within ±2 chromosomes.

2.3.4 Live/dead staining

To quantify viability of cells in suspension, a Live/Dead® kit [Invitrogen L3224], containing two reagents, was used. The first reagent, calcein AM, is cell permeable and produces the fluorescent green dye calcein when exposed to intracellular esterase activity, ubiquitously present in live cells. Calcein is polyanionic and so is well retained within cells when produced intracellularly. The second reagent, ethidium homodimer-1, is excluded from intact cells, but will enter cells whose membranes have been disrupted, and fluoresce bright red when bound to DNA. When reagents are at the optimum

concentrations, all cells incubated with a mixture of the two will appear either green or red under a fluorescence microscope.

Since we wanted to determine the viability of cells within the methylcellulose suspension culture, and not any effects which may be due to the retrieval procedure, the reagents were incubated directly with the cells in a 50µl aliquot of methylcellulose removed from suspension culture, using a cut tip to minimise shearing. Both reagents were prepared at 1µM final concentration in PBS, and 100µl of the mixture was added on top of the cells in methylcellulose on a standard microscope slide. The slides were incubated in a Petri dish at 37°C for 30 minutes, then samples were covered with large coverslips and viewed immediately under the fluorescence microscope.

2.4 p27 knockdown approaches

2.4.1 p27 siRNA design and transfection

Four different commercially designed siRNA duplexes were used [HP GenomeWide siRNA, Qiagen]. These have been designed to specifically target the rat sequence of the p27 gene CDKn1b.

```
1) Target sequence:   CAG CTC CGA ATT AAG AAT AAT
siRNA duplex:           G CUC CGA AUU AAG AAU AAU
                        | ||| ||| ||| ||| ||| |||
                        C GAG GCU UAA UUC UUA UUA

2) Target sequence:   CAG TTA ATT GTT TAG CGG TAA
siRNA duplex:           G UUA AUU GUU UAG CGG UAA
                        | ||| ||| ||| ||| ||| |||
                        C AAU UAA CAA AUC GCC AUU

3) Target sequence:   CTG AAT TCT TAG AAT TGA CTA
siRNA duplex:           G AAU UCU UAG AAU UGA CUA
                        | ||| ||| ||| ||| ||| |||
                        C UUA AGA AUC UUA ACU GAU
```

4) Target sequence: TCG GTG AGA ACT GAT CCT TTA

siRNA duplex: G GUG AGA ACU GAU CCU UUA

| ||| ||| ||| ||| ||| |||

C CAC UCU UGA CUA GGA AAU

siRNA stocks were kept at 20μM in Qiagen's proprietary 'siRNA suspension buffer', as aliquots frozen at -20ºC, and thawed as needed. Duplexes were incubated for 10 minutes at room temperature in DMEM [Gibco 11880] with HiPerfect Reagent [Qiagen] added at 1:18 dilution to form siRNA/lipid complexes for delivery to cells. Different final concentrations of siRNA, from 0.5nM to 10nM, were used, either a single sequence or a mixture of two. After adding siRNA complexes to cells growing on 6-well plates and leaving overnight, the medium on cells was changed, and 24 hours later, samples were harvested for Western blot. p27 levels were compared in cells that had received targeted siRNA to those that had been transfected with an equal amount of non-targeted negative control siRNA [Qiagen cat. no. 1022076]:

Negative control siRNA: UUC UCC GAA CGU GUC ACG U

||| ||| ||| ||| ||| ||| |

AAG AGG CUU GCA CAG UGC A

This sequence has no known homology to any mammalian gene according to the manufacturer, and so controls for non-specific effects on cells such as siRNA toxicity.

2.4.2 p27 shRNA design and generation of cell lines

Selection of target sequences and vector cloning:

Three different short hairpin RNAs (shRNAs) were designed using Clontech's 'RNAi target sequence selector' online tool, and a fourth was taken from the literature (Itoh et al. 2007), having been successfully used to target p27 in mouse cells (in a region where the target sequence is conserved between

mouse and rat), using a similar vector. The p27 gene sequence used to design targets can be found at:

http://www.ensembl.org/Rattus_norvegicus/Gene/Sequence?db=core;g=ENSRNOG00000007249;r=4:171841696171846572;t=ENSRNOT00000049848;time=1227634574090.09

Of the ten possible target sequences given by the program, numbers 2, 3 and 7 were chosen, on the basis that they are well separated from each other and thus will target different regions of the gene; and also that BLAST searches of each did not reveal sequence similarity to any other rat gene. The target sequences were used to design shRNA duplexes suitable for cloning into a retroviral expression vector using Clontech's 'shRNA sequence designer' online tool. This places the target sequence and its reverse complement arranged as a mirror image either side of the hairpin loop sequence TTCAAGAGA, and adds BamH1 and EcoR1 overhangs for insertion into the pSIREN vector, plus a Mlu1 restriction site for insert identification. The target sequences and resulting shRNA duplexes are shown below, with the target in pink, its reverse complement in green, and the Mlu1 site highlighted in yellow.

Target "2": TGCCGAGATATGGAAGAAG

```
5'-gatccGTGCCGAGATATGGAAGAAGTTCAAGAGACTTCTTCCATATCTCGGCATTTTTTTACGCGTg-----3'
3'-----gCACGGCTCTATACCTTCTTCAAGTTCTCTGAAGAAGGTATAGAGCCGTAAAAAATGCGCActtaa-5'
```

Target "3": GCTTTAGTTCTGGGAGATC

```
5'-gatccGCTTTAGTTCTGGGAGATCTTCAAGAGAGATCTCCCAGAACTAAAGCTTTTTTACGCGTg-----3'
3'-----gCGAAATCAAGACCCTCTAGAAGTTCTCTCTAGAGGGTCTTGATTTCGAAAAAATGCGCActtaa-5'
```

Target "7": GAGGTAGTGGGTTGATCAT

```
5'-gatccGAGGTAGTGGGTTGATCATTTCAAGAGAATGATCAACCCACTACCTCTTTTTTACGCGTg-----3'
3'-----gCTCCATCACCCAACTAGTAAAGTTCTCTTACTAGTTGGGTGATGGAGAAAAAATGCGCActtaa-5'
```

Target “itoh”: GTGGAATTTCGACTTTCAG

```
5'-gatccGTGGAATTTCGACTTTCAGTTCAAGAGACTGAAAGTCGAAATTCCACTTTTTTACGCGTg-----3'
3'-----gCACCTTAAAGCTGAAAGTCAAGTTCTCTGACTTTCAGCTTTAAGGTGAAAAAATGCGCActtaa-5'
```

The shRNA duplexes were purchased from Sigma and cloned into the retroviral expression vector RNAi-Ready pSIREN-RetroQ-ZsGreen [Clontech cat. no. 632455] between the BamH1 and EcoR1 sites as directed. Preparation of the shRNA expression vectors was carried out according to the manufacturer’s instructions:

1) shRNA duplexes were annealed by mixing 100µM of each oligonucleotide in a 1:1 ratio and heating to 95ºC for 30 seconds to disrupt the secondary hairpin structure, followed by gradual cooling over 6 minutes.
2) Annealed shRNAs were diluted to 0.5µM concentration (a 1:100 dilution) in TE buffer, and ligated to the linearised vector by incubating the following mixture at room temperature for 3 hours:

2µl	pSIREN-RetroQ-ZsGreen vector (25ng/µl)
1µl	shRNA oligonucleotide (0.5µM)
1.5µl	T4 DNA ligase buffer (10x)
0.5µl	BSA (10mg/ml)
9.5µl	nuclease-free water
0.5µl	T4 DNA ligase enzyme (400U/ml)
15µl	

A ligation reaction was set up as above for each of the four p27 target oligonucleotides, plus a vector-only control.

3) Each completed ligation reaction was transformed into competent HB101 bacteria by adding 2µl ligation mixture to 50µl of bacterial cell suspension, incubating on ice for 5 minutes and then heat shocking at 42ºC for 30 seconds in a water bath before replacing on ice.
4) Transformed bacteria were grown up in 250µl SOC medium shaking for 1 hour at 37ºC, and then 30µl of each transformation was spread onto

agar plates containing the selection antibiotic ampicillin under aseptic conditions, and incubated at 37ºC overnight.

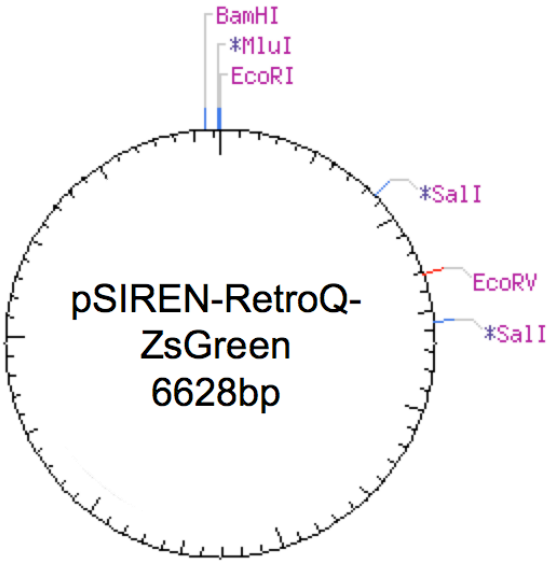
5) 3 or 4 well separated colonies from each selection plate were isolated and grown up in small starter cultures of LB medium + ampicillin for 8 hours, then plasmids were purified from the bacteria and digested to check for the presence of the shRNA insert (see below).
6) Once the presence of an insert was confirmed by restriction digest, 0.5ml of the starter culture was inoculated into 250ml LB medium + ampicillin and grown up at 37ºC overnight with vigorous shaking. Plasmid was purified from this large bacterial culture by maxi prep [Qiagen] and kept as a stock dissolved in TE and frozen at -20ºC.

Diagnostic restriction digests to check for shRNA vector inserts:

Restriction digests to check for the presence of an Mlu1-containing insert were carried out on plasmid from 3-4 separate colony cultures for each of the four inserts, and also on the re-ligated vector alone (no insert) and a control plasmid containing a negative control insert [Clontech] (half the hairpin sequence against a luciferase target gene):

```
5'   GAT CCG TGC GTT GCT AGT ACC AAC TTC AAG AGA TTT TTT ACG CGT G    3'
          GC ACG CAA CGA TCA TGG TTG AAG TTC TCT AAA AAA TGC GCA CT TAA
```

A representative diagram showing the relative positions of the restriction sites in the vector is shown below. The insert is located between the BamH1 and EcoR1 sites, and all inserts used contained a unique Mlu1 site as shown.



Five different restriction digests were set up: the first three single digests were only carried out as controls on one vector; the two double digests were carried out on all vectors as diagnostic for the presence of a Mlu1-containing shRNA insert.

(i) EcoRV single digest

One predicted fragment: 6628bp

(ii) Sal1 single digest

Two predicted fragments: 5927bp + 701bp

(iii) Mlu1 single digest

One predicted fragment: 6628bp

(iv) Mlu1 + EcoRV double digest

Two predicted fragments in vectors with an insert: 5301bp + 1327bp (only one 6568bp fragment if insert is missing)

(v) Sal1 + Mlu1 double digest

Three predicted fragments in vectors with an insert: 5066bp + 861bp + 701bp (only two 5867bp + 701bp fragments if insert is missing).

The four p27 shRNA plasmid stocks from the maxi prep, having been checked at the mini prep stage for Mlu1 sites by restriction digest (see Figure 4-7), were further verified by sequencing. The U6 promoter sequence

5′-ATGGACTATCATATGCTTACCGTA-3′

(located approximately 50bp upstream of the insert site) was used as a forward sequencing primer, as suggested in the instructions accompanying the pSIREN-RetroQ-zsGreen vector, and sequencing was carried out by MWG Biotech. Plasmids with insert targets "2", "3" and "itoh" were 100% matched to their correct insert sequence, while insert target "7" was sequenced correctly for the first 18 nucleotides before the read failed – probably due to the confounding secondary structure of the hairpin.

Diagnostic PCR to verify expression of correct vectors

Genomic DNA was purified from the sorted zsGreen cells using the DNeasy spin-column protocol [Qiagen] and a diagnostic PCR was performed. Primers were designed to detect the presence of the control shRNA insert in the pSiren vector, using the Primer3 designer program:

http://frodo.wi.mit.edu/

All options were kept at default, except that the reverse primer was obligatorily targeted to the negative control insert sequence (Figure 2- 4).

2.5 In vivo tumourigenesis assay

20 female CD-1 nude mice at 35-41 days old were obtained from Charles River and housed in individually ventilated cages using sterile supplies. NSLT cells from 72-hour suspension culture, which had been replated and passaged twice, were injected subcutaneously into 9 mice, once on each flank (2 injections per mouse, performed under gas anaesthetic). Injections were carried out using 21G sterile needles, and approximately 750,000-1 million cells in 150-200µl sterile PBS were introduced per flank. Another 9 mice were injected in the same way with NSLT negative control cells, which had been briefly introduced to methylcellulose suspension in parallel with the test cells, but were immediately retrieved and replated. 2 mice were injected with NSLTRas cells as a positive control, and were sacrificed after 12 days due to the growth of subcutaneous tumours up to 10mm in diameter on both flanks. The remaining mice were monitored for signs of tumour growth. After 4 months, one negative control and one test mouse were culled due to sickness unrelated to tumour formation. Injections and culls were carried out by Drs L. Noon and I. Napoli.

```
LEFT PRIMER (1):                    cccatgattccttcatatttgc
LEFT PRIMER (2) [U6 promoter]:      atggactatcatatgcttaccgta
RIGHT PRIMER (reverse complement):  tagcaacgcacggatcctc

PRODUCT SIZES: (1) 248bp; (2) 92bp

    1
gaacctcctcgttcgaccccgcctcgatcctccctttatccagccctcactccttctcta

   61
ggcgccggaattgaagatctgggcaggaagagggcctatttcccatgattccttcatatt

>>>>>>>>>>>>>>>>>>>

  121
tgcatatacgatacaaggctgttagagagataattagaattaatttgactgtaaacacaa
      >>>

  181
agatattagtacaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagttt

  241
taaaattatgttttaaaatggactatcatatgcttaccgtaacttgaaagtatttcgatt
                       >>>>>>>>>>>>>>>>>>>>>>>>

  301
tcttggctttatatatcttgtggaaaggacgaggatccgtgcgttgctagtaccaacttc
                                    <<<<<<<<<<<<<<<<<<<

  361 aagagatttttacgcgtg


KEYS:
>>>>>> left primer
<<<<<< right primer
```

Figure 2- 4: Positioning of PCR primers to identify negative control insert in pSIREN-RetroQ-zsGreen vector.

Reverse primer is obligatorily targeted to the negative control insert (highlighted in green). The two alternative forward primers are expected to produce PCR products of 248bp and 92bp respectively, only in genomic DNA from cells expressing the negative control insert (see Figure 4-8B). Diagram copied from output of Primer3 online program.

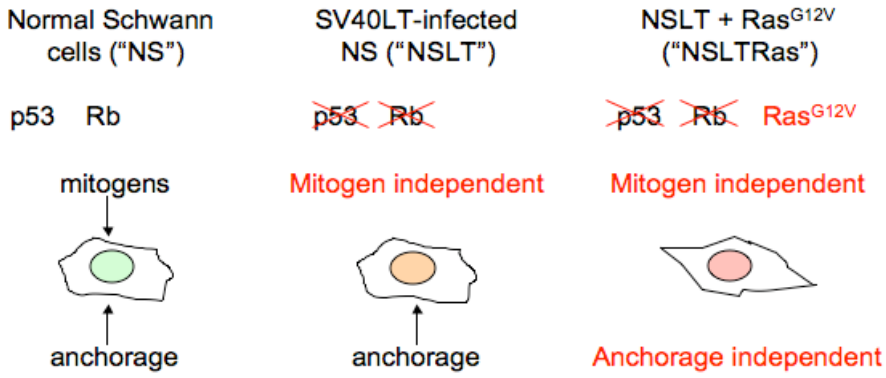
Chapter Three – Characterisation of a primary cell model showing loss of anchorage dependence for proliferation

3.1 Chapter introduction

A key property of transformed cells in vitro and those which are malignant in vivo is the ability to divide regardless of support from the surrounding matrix, a characteristic known as anchorage-independent proliferation. In order to investigate how the genetic changes associated with cancer can permit cells to proliferate in the absence of anchorage signals, we chose to use a Schwann cell model system in which a single genetic change is sufficient to switch cells between anchorage dependent and independent proliferation (Mitchell et al. 2003).

Primary rat Schwann cells can be cultured under conditions in which they are immortal, yet with no loss of cell cycle checkpoints (Mathon et al. 2001). Use of the undifferentiated Schwann cell model enables us to introduce defined oncogenic changes step by step in a controlled way, with minimal risk of additional spontaneous mutation. This property is of the utmost importance when studying cell cycle regulation, as spontaneous genetic changes leading to immortalisation often affect the cell cycle, and this could lead to confusing results in a less genetically stable system. We chose to model the transformation process using (1) the viral oncoprotein Large T (LT), which binds and inactivates both p53 and the Rb family of pocket proteins: pRb, p107 and p130 (Lane and Crawford 1979; DeCaprio et al. 1988; Dyson et al. 1989; Hannon et al. 1993); and (2) the oncogenic Ras protein that harbours a G12V mutation, which traps the protein in its active GTP-bound form and causes constitutive activation of its many downstream targets (McGrath et al. 1984). Expression of both LT and oncogenic Ras proteins together has been shown to be sufficient for full transformation, including anchorage independence, in mouse and rat cells (Michalovitz et al. 1987; Clark et al. 1988; Hirakawa and Ruley 1988; Ridley et al. 1988; Beachy et al. 2002); expression of hTERT is additionally required in human cells, to satisfy the requirement for immortalisation (Hahn et al. 1999). Normal Schwann cells expressing LT alone

(NSLT) remain anchorage dependent, despite losing their requirement for mitogens.



Since previous evidence suggests that anchorage signals act in combination with mitogens to alleviate the Rb-E2F checkpoint (see Introduction 1.1.3.1) we might expect the inactivation of all the Rb family pocket proteins to remove the requirement for anchorage signals as well as mitogens for proliferation. The fact that it does not, demonstrates that anchorage signals are also required downstream of Rb inactivation to drive cell cycle progression. We previously showed that p53 inactivation is required to cooperate with oncogenic Ras to induce proliferation in these cells (Mitchell et al. 2003), consistent with studies in other cell types (Hicks et al. 1991; Fukasawa and Vande Woude 1997; Azzoli et al. 1998), but this was not sufficient to confer anchorage independence. NSLT cells therefore appear to exhibit a novel anchorage checkpoint, which is independent of both Rb and p53 function. The 'switch' to anchorage independent proliferation upon additional expression of oncogenic Ras in these cells (NSLTRas) shows that only one genetic change is required to alleviate this secondary anchorage checkpoint, and the downstream effects of constitutive Ras activation may give clues to its mechanism. New NSLT and NSLTRas cells were created in parallel, to ensure a carefully-controlled model cell system, and initial experiments were designed (a) to set up optimal conditions for suspension culture, allowing the biochemical analysis of both attached and suspended cells, and (b) to characterise the cell cycle in normal Schwann cells (NS), NSLT, and NSLTRas cells, both when attached and in suspension.

3.2 Genetic construction of model cell types

3.2.1 Infection of cells

It was considered important to use low-passage cells of equivalent 'age' to create stock populations of "NSLT" and "NSLTRas" that allow unbiased comparison between these cell types in subsequent experiments. Recently-established, low passage rat Schwann cell cultures expressing LT Babe-Puro were infected in parallel with either 'empty' LXSN retroviral vector or Ras LXSN, containing the H-Ras^{G12V} oncogene (see Materials and Methods). Retroviral infection of these cells is highly efficient, resulting in pools of cells that maintain a high complexity of vector expression, rather than clones. The infected NSLT and NSLTRas populations were drug-selected with G418 to ensure all cells were expressing the vector. Although normal Schwann and LT-expressing Schwann cells are phenotypically relatively stable in culture under carefully-controlled conditions (Porter et al. 1986; Ridley et al. 1988; Mathon et al. 2001; Mitchell et al. 2003), cells drug-selected for LXSN expression were expanded and kept as low-passage stocks, which were then used at equivalent passage in experiments up to a maximum of 20 passages, to control for any inconsistency in cell behaviour in suspension culture that may occur after too many population doublings.

3.2.2 Cell morphology and LT expression

The morphology of the three cell types in normal attached culture conditions shows that while NSLT cells resemble normal Schwann (NS) cells, i.e. flattened, with an opaque nucleus; NSLTRas cells have a more elongated shape and appear refractive: a typical 'transformed' cell morphology indicative of oncogenic Ras expression (Figure 3- 1). NS cells divide more slowly than the NSLT and NSLTRas cells, and are therefore seeded at a higher density in order to reach 80% confluence for passaging within three days. All three cell populations maintain a homogeneous appearance throughout culture and passaging.

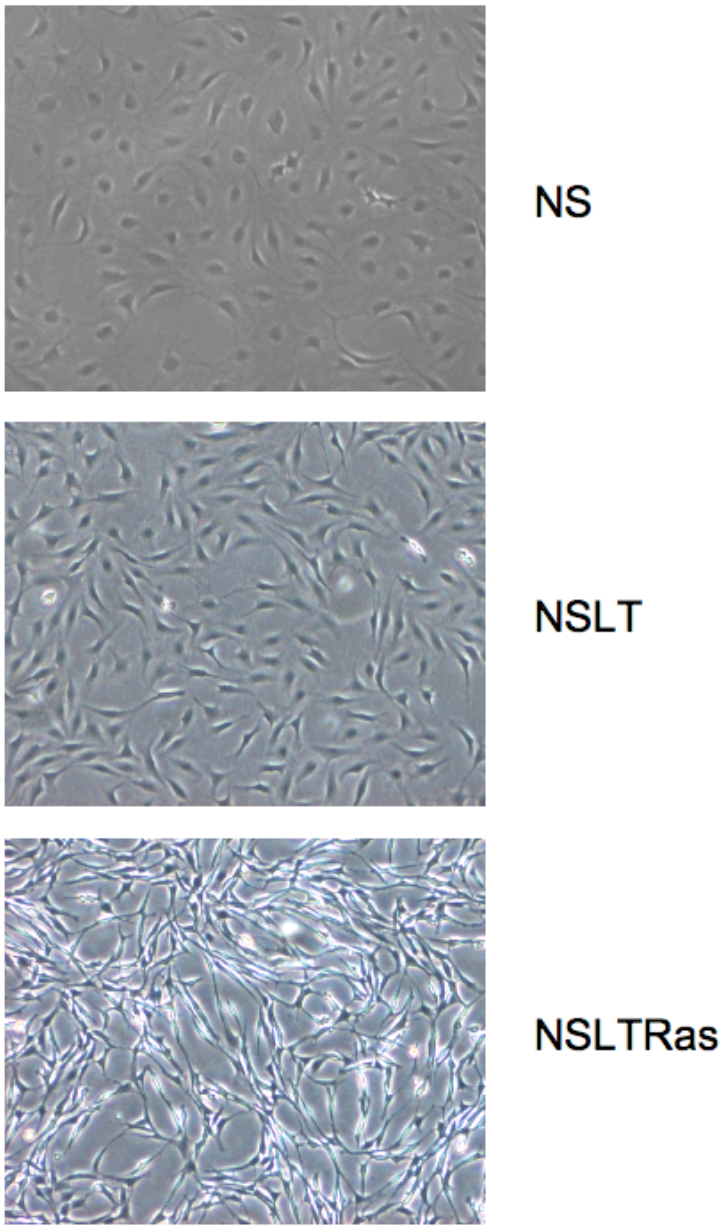


Figure 3- 1: Morphology of model cell types.

Phase contrast micrographs of NS, NSLT and NSLTRas cell cultures on PLL-coated plates. NS and NSLT cells appear more rounded and flattened, while NSLTRas cells have a more elongated shape and appear highly refractive. X4 objective.

In order to assess LT expression levels in the newly constructed cell stocks, cell samples were cultured on coverslips, fixed, and stained with a LT antibody (Figure 3- 2A). Observation by immunofluorescence showed nuclear expression of LT protein in both NSLT and NSLTRas cells, but not in NS cultures. Quantification of LT expression indicated that at least 99% of cells in both NSLT and NSLTRas populations were positive for LT staining (Figure 3- 2B). Moreover, the intensity of fluorescence was similar in NSLT and NSLTRas nuclei, indicating similar expression levels in the two cell types.

3.3 Cellular Characterisation

3.3.1 Colony formation in soft agar

To assess colony-forming ability in our newly constructed model cell types, NS, NSLT and NSLTRas cells were seeded into soft agar to assay anchorage-independent proliferation. Seeding cells in 6-well plates at three different densities (2000, 4000 and 8000 cells/well) and staining cells with MTT after two weeks clearly showed that only NSLTRas formed colonies, and both NS and NSLT cells remained anchorage dependent (Figure 3- 3). The average colony size correlated inversely with cell density, indicating that the availability of mitogens and growth factors was limiting after some days within the agar, despite regular additions of fresh medium to the top of the plate. Stained NSLTRas colonies were large enough to be seen with the naked eye, while microscopic inspection of the NS and NSLT plates revealed the persistence of single cells within the agar (Figure 3- 4A).

In order to more closely observe the anchorage-independent proliferation leading to colony formation, and to see what happens to the anchorage dependent cell types while existing as single cells in suspension, NSLT and NSLTRas cells were seeded in very thin (<3mm) layers of soft agar and viewed by time-lapse microscopy. Cultures were viewed over a twenty-four hour period, during which time 4-8 focus points were set up on individual cells within the suspension and images captured every 15 minutes. The resulting movies showed that these cells, though rounded and non-invasive,

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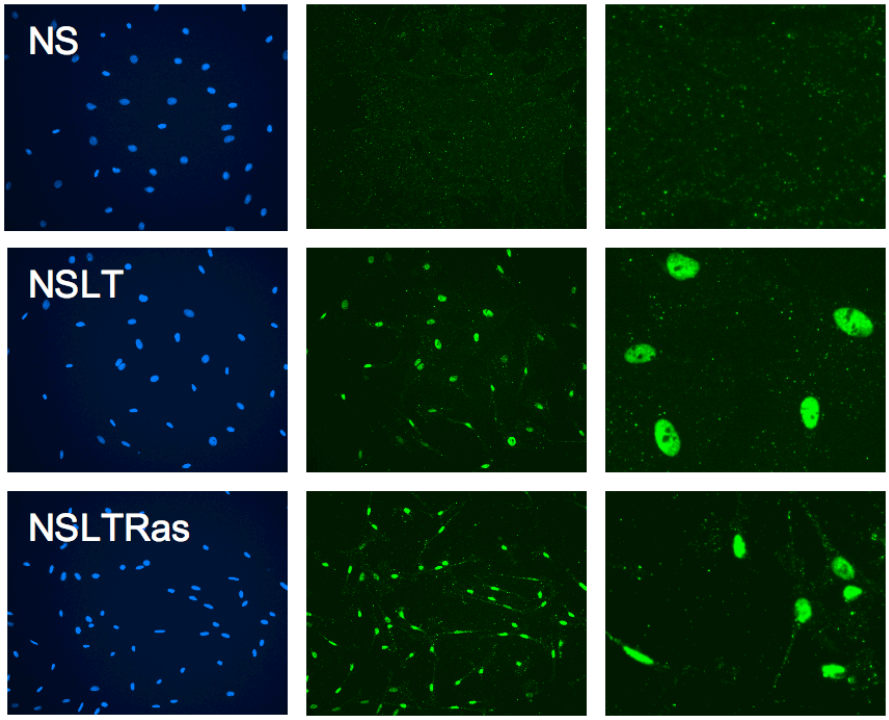

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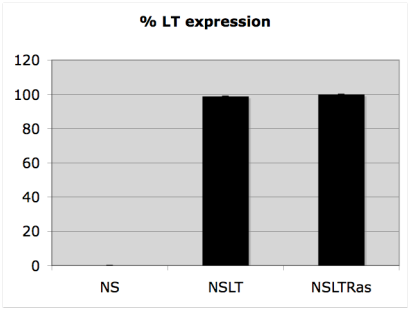
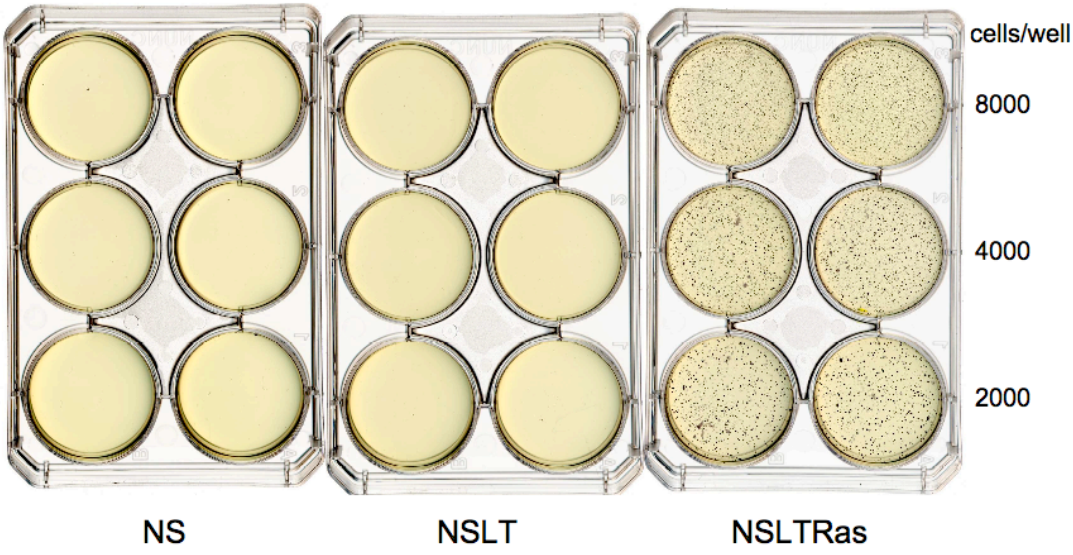


Figure 3- 2: Nuclear SV40 LT antigen expression in NSLT and NSLTRas cells.

(A) Fluorescence micrographs of NS, NSLT and NSLTRas cells seeded at 20,000 cells/coverslip and immunostained the following day with polyclonal Ab419 (1:50) against the LT N-terminus, Alexa Fluor 488 anti-mouse fluorescent-conjugated secondary antibody (1:300) and Hoechst (1:5000). Left panels show Hoechst-stained DNA (blue); middle and right (magnified) panels show nuclear LT staining (green). X25 objective.

(B) Quantification of LT-positive cells in each culture. Nuclei were counted on 3 coverslips per cell type.

A



B

Figure 3- 3: Soft agar assays of NS, NSLT and NSLTRas cells.

(A) Cells were seeded in soft agar at 2000, 4000 or 8000 cells/well and incubated for 2 weeks, fed weekly, before staining overnight with MTT [Calbiochem 475989, 10mg/ml; 100µl/well].

(B) Enlarged wells from the above photograph, showing the 2000-cell seeding density. NSLTRas (right) show colonies visible to the naked eye; no colonies are visible in either the NS (left) or NSLT (middle) cultures.

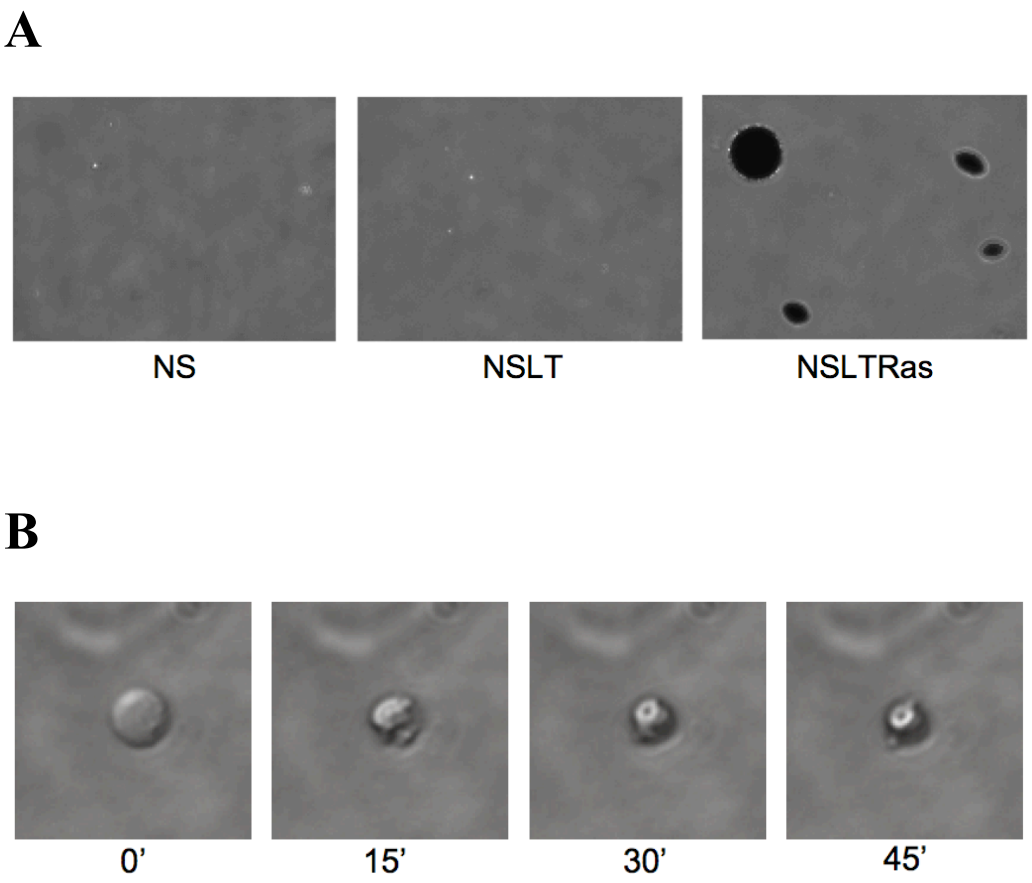


Figure 3- 4: NS and NSLT appear as single cells in soft agar suspension.

(A) Cells were seeded in 0.6% soft agar suspension in 6-well plates and viewed by phase contrast microscopy after two weeks. Individual NS and NSLT cells can be seen as round white dots, while MTT-stained NSLTRas colonies are black. X4 objective. (B) 4000 NSLT cells were seeded in 500µl 0.6% agar incubated in a thin layer in a 12-well plate. Shown are stills from time-lapse microscope photography of a single cell. Cell morphology changes from fully rounded and pale to collapsed and dark, indicating a cell death. X25 objective.

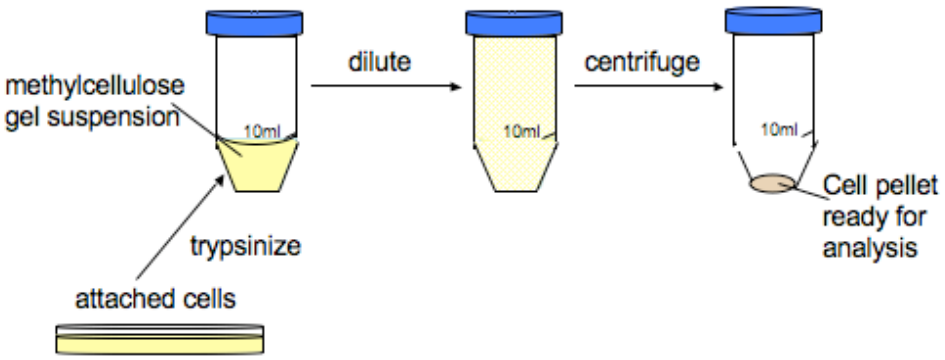
continually change shape while in soft agar suspension, strong evidence that they remain alive even when not dividing. This finding was supported by one movie that appeared to show cell death – the NSLT cell in question changed from a shiny, fully-rounded morphology to a collapsed, dark-looking speck within the space of 45 minutes (Figure 3- 4B). Based on these and previous observations of cells in soft agar in the lab, this bright, reflective and non-'blebbed' appearance was taken to indicate a live cell. In larger soft agar cultures, cells overwhelmingly maintain the 'live' morphology for at least four weeks if kept fed, and only started to appear blebbed and shrunken if medium was omitted.

The time-lapse method however proved unhelpful for observing NSLTRas cell division, as the optics of the medium were insufficiently sharp to reveal a clear cytokinesis, and cells tended to move out of the field of view as the agar contracted in the viewing chamber (despite humidifying the plate by overlaying the agar with medium and filling unused wells with sterile water). Following these observations, this technique was abandoned, as the optical limitations were too great to allow more detailed imaging, for example the staining of mitotic chromosomes.

3.3.2 Optimisation of methylcellulose seeding and retrieval

In order to obtain enough cells for protein analysis, even from anchorage-dependent samples in disperse suspension culture, an alternative to soft agar was required, as retrieval of cells from soft agar cultures would necessitate the isolation of individual colonies by pipette. Methylcellulose gels have previously been used in suspension culture, and were not inhibitory to cell growth and proliferation (Stoker et al. 1968; Otsuka and Moskowitz 1975). This was confirmed in our system by overlaying an ordinary plate of NS cells with medium containing 2% methylcellulose - no change was observed in their morphology or proliferation (judged by the time taken for a plate to reach confluence). The great advantage of using this type of gel over soft agar is that it can be diluted and the cells retrieved by centrifugation. Different methylcellulose concentrations and centrifugation speeds were tested in order to determine the conditions allowing the retrieval of most intact cells, while not

allowing cells to sink to the bottom of the suspension culture. Stoker et al. found that methylcellulose concentrations below 1% were too low to prevent cells aggregating in suspension, but had difficulty pipetting the gel at concentrations above 1.5%. They settled on a methylcellulose concentration of 1.3%, which allowed cells to remain discrete with acceptable ease of handling. However, they observed that cells close to the sides and base of the dish were able to adhere to and spread on the surface, and overcame this problem by coating culture ware with agar before adding the methylcellulose-cell suspension. Despite this precaution, the authors reported that cells 'fell slowly through the gel and eventually came to lie on the agar surface', although this interface did not support cell spreading. In order to retrieve cells for analysis the methylcellulose suspension was taken up into a wide-bore pipette and diluted with medium, washing the surface of the agar to retrieve all the cells (Stoker et al. 1968). For our purposes, we used the simpler method of incubating the methylcellulose suspension directly in centrifuge tubes, allowing immediate addition of diluting medium without the need for pipetting of the viscous gel:



This method has previously been described using silicone-coated test tubes (Otsuka and Moskowitz 1975). As our method could be accomplished by pouring and no longer required pipetting of the gel, higher concentrations of methylcellulose medium could be used to ensure that cells were adequately supported in suspension and did not sink or spread on the walls of the tube. We tested concentrations of 1.5% and 1.8%, and found that a minimum concentration of 1.5% was sufficient to support cells - tested by taking small

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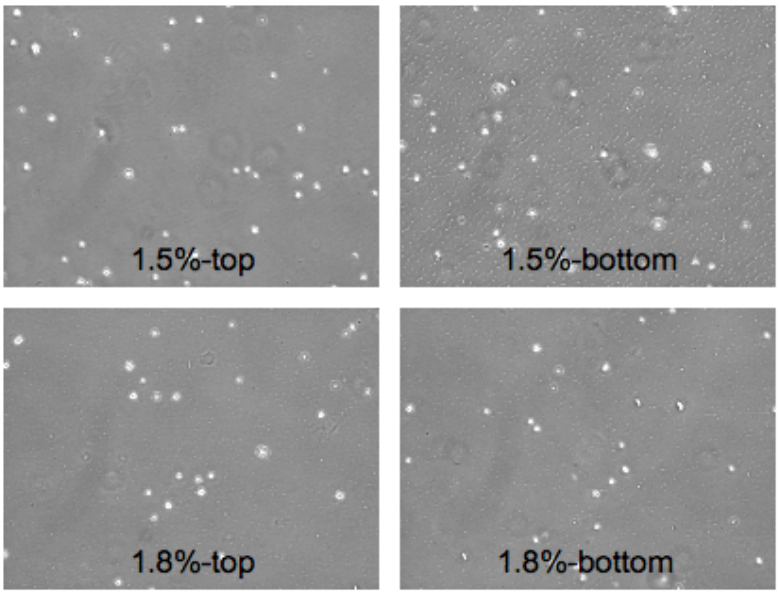

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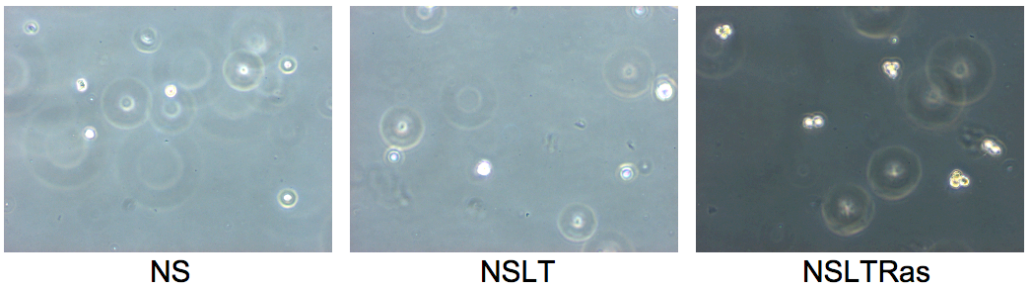

Figure 3- 5: Cells remain evenly distributed in methylcellulose suspension.

(A) NSLT cells were seeded in 1.5% and 1.8% methylcellulose suspension and mixed thoroughly by inversion. Aliquots were taken from the top and bottom of the culture tube after 24 hours, to check for even cell distribution. X4 objective.

(B) NS, NSLT and NSLTRas cells were seeded in 1.8% methylcellulose suspension. Aliquots were taken from the cultures after 2 days and viewed by phase contrast microscopy. NS and NSLT appear as single cells, and NSLTRas as 2-4 cell 'colonies'. Cells all appear brightly refractive, indicating that they are viable. X4 objective.

aliquots from the top and bottom of the tube after 24 hours' incubation and comparing the density of cells in each position (Figure 3- 5).

Methylcellulose gel was made up at 1.8%, which following addition of trypsinised cells at 1:9 gave a final dilution of 1.62%. After 2 days in methylcellulose, differences in cell proliferation could be seen under the microscope (Figure 3- 5), with NSLTRas cells visible as small colonies of 2-4 cells, whereas NS and NSLT samples remained as single cells. In preliminary retrieval experiments from 2% methylcellulose, centrifuging at 650 *g*, the number of intact cells recovered fell after 24 hours in suspension (Figure 3-6A), so we tested lower spin speeds of 500 and 250 *g* to see whether this would improve recovery. Recovery numbers were in fact highest at 500 *g*, retrieving around 95% of cells immediately after seeding, and 75% 24 hours later, a figure that was not improved by varying the methylcellulose concentration (Figure 3-6B).

Recovery procedures were further refined by varying the washing and trypsinising steps before cell counts were made. Using relatively small volumes of methylcellulose ($1/5^{th}$ the tube volume) allowed space within the tube for both equilibration with the CO_2-controlled atmosphere within the incubator, and for addition of dilution liquid when retrieving. DMEM plus 1% serum was used to dilute the suspension and helped stabilise the cells before washing with PBS. It was found that warm methylcellulose did not mix uniformly with cold solution, so warm DMEM +1% serum was initially used to dilute the suspension 1:2 before addition of further ice-cold solution. Samples were incubated on ice while harvesting, to preserve the integrity of cells as far as possible. After washing, trypsin was used to disperse cells before counting. However, it soon became clear that using the same procedure for the different cell types did not result in quantitative recovery. Simply resuspending the washed pellet in trypsin, before addition of serum-containing medium to quench the enzyme activity, was not sufficient to separate tightly aggregated NSLTRas cells, which showed up on the Coulter counter as large clumps. Passing the suspension through an 18G needle was successful in separating these clumps and increased the effective NSLTRas count. However, this treatment drastically reduced the counts from NSLT cells, which appeared on the Coulter counter as

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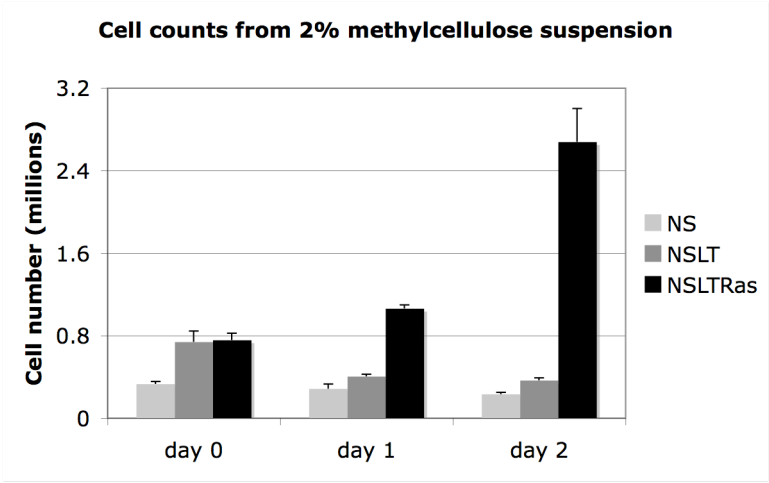

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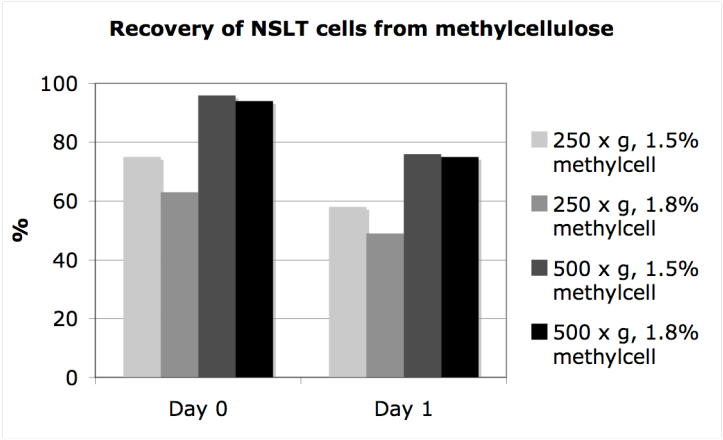


Figure 3- 6: Optimisation of methylcellulose cell retrieval procedure.

(A) 800,000 NS, NSLT and NSLTRas cells were seeded in 2% methylcellulose and retrieved by 650 *g* centrifugation on the day of seeding (day 0), and after 1 and 2 days. Graph shows mean of duplicate cell counts for each cell type. Error bars indicate s.d. (B) NSLT cells were seeded in 1.5% or 1.8% methylcellulose and retrieved by centrifugation at either 250 or 500 *g*, aiming to improve efficiency of cell retrieval. Graph shows percentage recovery for each condition.

debris, suggesting that not only were NSLT cells not dividing to form colonies, they are also less physically robust in suspension. It was therefore decided to use a gentle resuspension by pipette for NS and NSLT cells, and a more stringent resuspension using the 18G needle for NSLTRas cells, to ensure the production of a single-cell suspension for counting.

3.3.3 NSLTRas proliferate in suspension, but NS and NSLT do not

Counts of retrieved, washed and dispersed cells pelleted from suspension culture (Figure 3- 7) showed a clear increase in numbers of NSLTRas cells over time, quadrupling in number over 48 hours. This is similar to their normal 24-hour cell doubling time in attached culture, showing that NSLTRas cells proliferate as well in suspension as they do on plates. In contrast, NS and NSLT cells did not increase in number over 48 hours, and the number of NS cells retrieved decreased over time. It was thought that this was partly due to cells being less robust in withstanding the tough retrieval and washing procedure, since NSLTRas could withstand fairly vigorous syringing through an 18G needle to disperse cells from their tight colonies, whereas even gentle resuspension of NS samples resulted in fragmentation of the cells. This may account for the less than 100% retrieval rate of this cell type.

3.3.4 The majority of cells survive in suspension

To quantify the cell loss that occurred due to cell death while in suspension, rather than due to loss or breakage during retrieval, a commercial kit that identifies both live and dead cells was used to stain small samples of cells in methylcellulose. Viability staining of suspended cells using this kit (Figure 3- 8A) revealed that a proportion of both NSLT and NSLTRas cells were dead in suspension, though most remained alive throughout the duration of the experiment (48 hours). This is consistent with Schwann cells' non-epithelial origin, which would suggest that they do not undergo automatic anoikis following detachment from the extracellular matrix (Frisch and Francis 1994). Expression of oncogenic Ras improved the NSLT cell survival rate from 74% to 87%, which may be due to the activation of PI3K-Akt pathways downstream of Ras (Khwaja et al. 1997). Early studies on the suspension of 3T3 fibroblasts

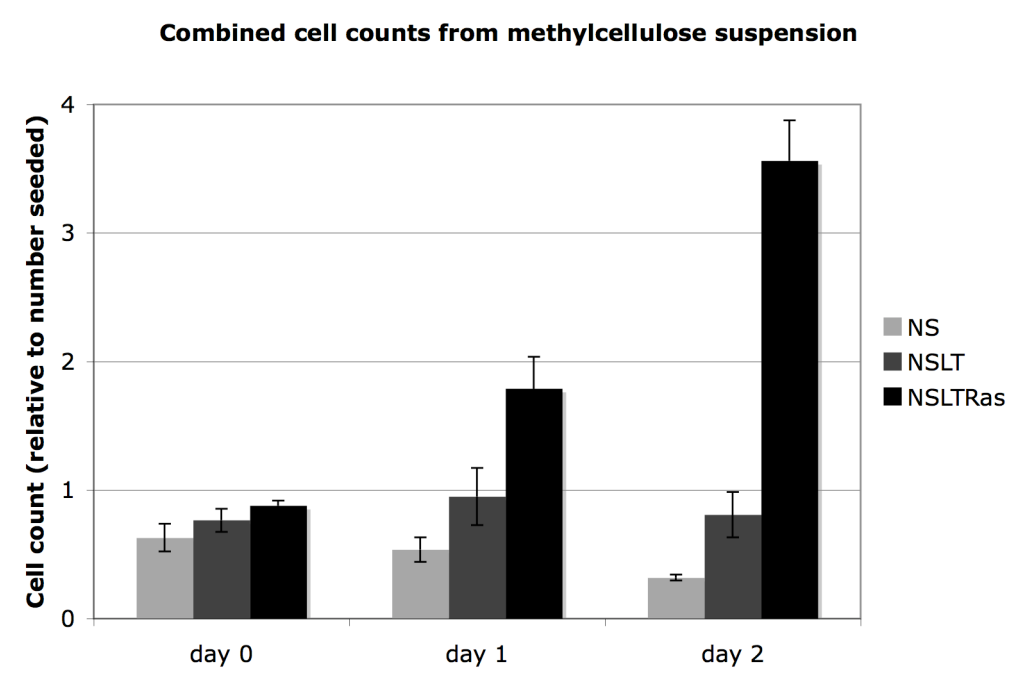


Figure 3- 7: NSLTRas cells increase in number in methylcellulose suspension, while NS and NSLT do not.

NS, NSLT and NSLTRas cells were seeded in 1.8% methylcellulose suspension and retrieved by centrifugation at 500 *g*. Duplicate samples were counted shortly after seeding (day 0), and then at approximately 24-hour intervals (day 1, day 2). NSLTRas cells roughly double in number every 24 hours (black bars), indicating anchorage-independent proliferation, while numbers of NS and NSLT cells retrieved do not increase over the course of the experiment (grey bars). Graph shows data combined from three similar experiments. Error bars indicate s.e.m.

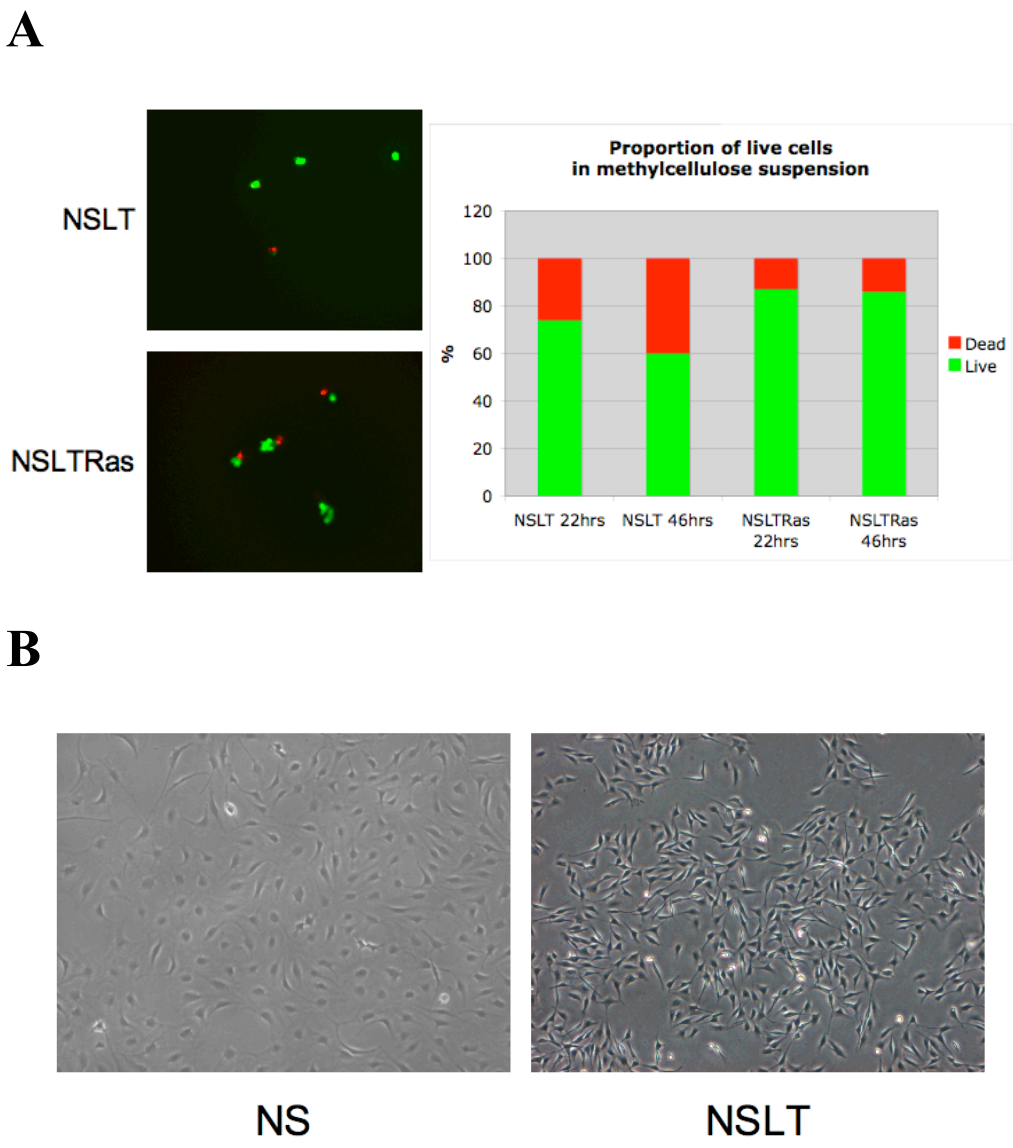


Figure 3- 8: Most cells remain viable in methylcellulose suspension and resume proliferating when retrieved and replated.

(A) Quantification of cell viability in methylcellulose suspension. Aliquots containing cells were removed from culture at the stated times and incubated for 30 minutes with components of the Live/Dead Viability/Cytotoxicity kit [Invitrogen]. Green indicates live cells; red cells are dead.

(B) Phase contrast micrographs of proliferating cells retrieved and replated from suspension culture. Left, NS cells replated after 30 hours; Right, NSLT cells replated after 36 hours in methylcellulose suspension.

found that trypan blue exclusion, which indicates live cells, was over 99% for at least 3 days in suspension (Otsuka and Moskowitz 1975). This is consistent with our observations of cells in soft agar. It is possible that the relatively low viscosity of methylcellulose compared to soft agar contributes to the reduced cell survival in this medium. This possibility will be discussed in Chapter Six.

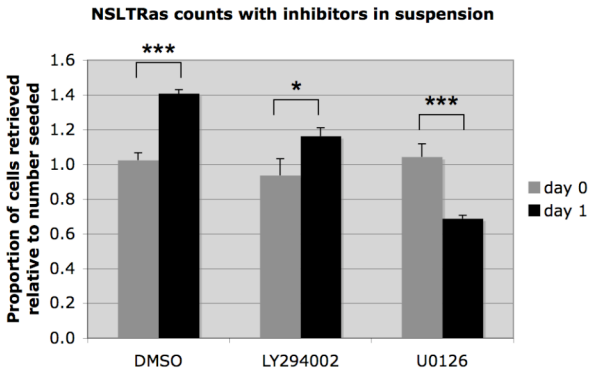
3.3.5 Cells replated from suspension culture resume proliferation

Further evidence to support the predominant survival of cells in suspension culture comes from the replating of cell samples recovered from suspension on ordinary culture plates (Figure 3- 8B). The majority of retrieved NS and NSLT cells reattached and resumed proliferation after several hours in methylcellulose as soon as they were allowed to settle, indicating a temporary, reversible arrest in suspension rather than a permanent cell cycle exit. This is consistent with the idea of a cell cycle 'anchorage checkpoint' in NSLT cells analogous to that in NS, which is alleviated when the requirement for attachment is met. NSLTRas cells likewise reattached as normal and continued their proliferation upon replating. This is consistent with previous studies, which showed that 3T3 fibroblasts exhibited a reversible cell-cycle arrest in suspension (Otsuka and Moskowitz 1975).

3.3.6 NSLTRas cells require Raf/ MEK to proliferate in suspension

Expression of oncogenic Ras in NSLT cells is sufficient to overcome their anchorage dependence, but there has not so far been a consensus on the downstream effectors responsible for this aspect of transformation (Yang et al. 1998; Repasky et al. 2004). In order to determine the pathways responsible in our system, we investigated whether the PI3K or ERK pathways, key mediators of Ras signalling, are necessary for NSLTRas cell proliferation in suspension. The inhibitors LY294002 and U0126 were added to methylcellulose containing NSLTRas cells. LY294002 inhibits PI3K, and U0126 inhibits the ERK upstream activating kinase MEK. As shown in Figure 3- 9A, cell counts indicated that NSLTRas were able to proliferate in the presence of LY294002, but not U0126, indicating that the ERK pathway, but not the PI3K pathway, was

A



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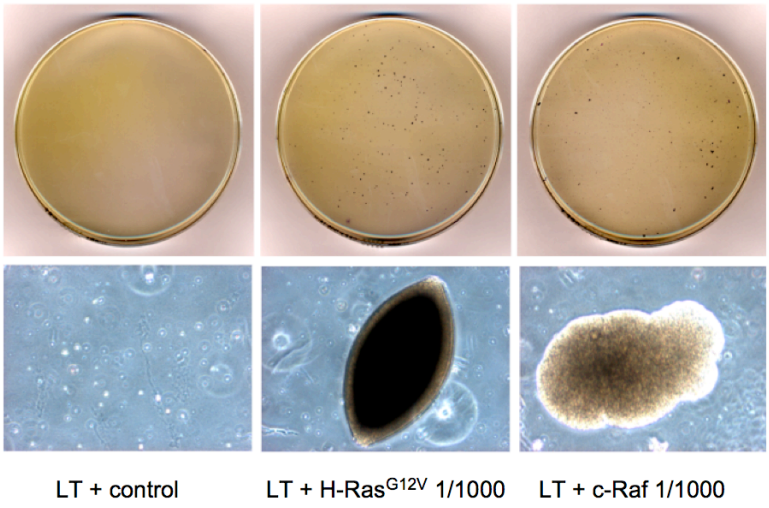

Figure 3- 9: Raf/MEK signalling is necessary and sufficient to overcome the anchorage checkpoint.

(A) NSLTRas cells were seeded in methylcellulose suspension containing either the PI3K inhibitor LY294002 (20μM), the MEK inhibitor U0126 (20μM), or DMSO (1:1000) as a vehicle control. Graph shows means of cell counts from two experiments. Error bars indicate s.e.m. * $p > 0.1$, no significant difference; *** $p < 0.02$, significant difference (2-tailed, unpaired t-test).

(B) LT-expressing cells were infected with either control vector (left panels), or a 1 in 1000 ratio of H-Ras^{G12V}- (middle) or c-Raf kinase domain-carrying vector (right panels) to control vector. Soft agar wells (top) show colonies in both the H-Ras^{G12V} - and c-Raf- expressing cultures visible to the naked eye. Phase contrast micrographs (bottom) show the morphology of H-Ras^{G12V} - and c-Raf-induced colonies. Experimental data from Davide Danovi.

necessary for Ras-induced anchorage independent proliferation. This result was supported by other studies (D. Danovi, personal communication), showing that the kinase domain of c-Raf (Ras-activated effector of the ERK pathway) was sufficient to induce colony formation in LT-expressing cells (Figure 3- 9B).

3.3.7 FACS analysis shows an aberrant cell cycle profile in NSLT suspended cells

Following the observations that NSLT cells do not proliferate in suspension, but are quickly able to return to the cell cycle once reattached, we performed flow cytometric analysis to determine in which phase the cells arrest, since this could give important clues to the biochemical mechanism of the anchorage checkpoint and how signals from the substratum feed into cell cycle control pathways. BrdU was added to both attached and suspended cultures one hour prior to harvesting. Samples were then fixed and stained with antibodies to BrdU, to mark cells actively progressing through S phase, and propidium iodide (PI), to indicate total DNA content. The results were gated to exclude cell aggregates and debris (Figure 3- 10A). Samples to which BrdU had not been added were fixed and stained in the same way, in order to define the BrdU-negative population and determine the threshold for identifying BrdU-positive cells (Figure 3- 10B).

Asynchronously dividing populations of attached NS, NSLT and NSLTRas cells exhibited a characteristic 'horseshoe-shaped' FACS profile, showing largely BrdU-negative clusters in G1 and G2/M, linked by an arc of BrdU-positive S phase cells (Figure 3- 11A). The relative proportions of cells in S and G2/M phases compared to G1 were increased in faster-cycling cells, reflecting a shortening of G1 phase (Figure 3- 11B). This could be seen in both NSLT (38% S/G2/M phase) and NSLTRas cells (41% S/G2/M) compared to NS (25%). Normal population doubling times in attached culture are around 24 hours for NSLT and NSLTRas cells, and around 48 hours for NS (Lloyd lab unpublished data).

After 24 hours in suspension, the FACS profile of NS cells showed a classical G1 arrest, with BrdU incorporation almost undetectable and nearly all

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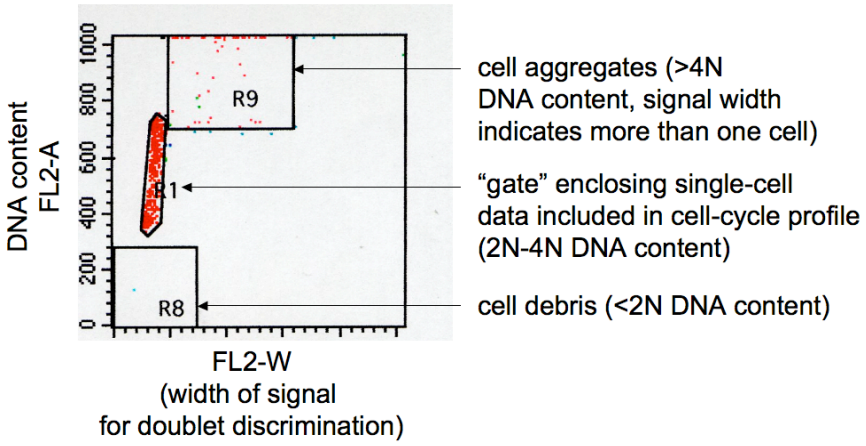

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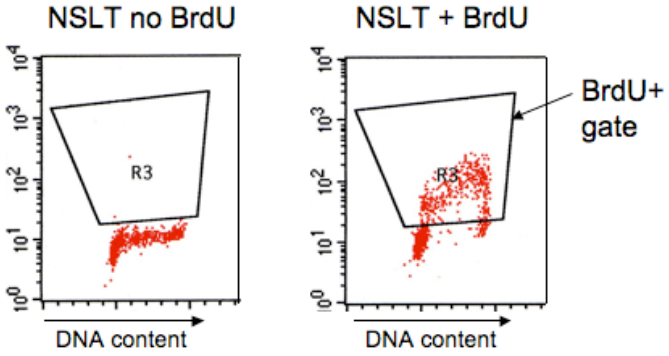

Figure 3- 10: Gates used for cell cycle analysis by flow cytometry.

(A) Dot plot from typical attached, proliferating cell sample, indicating gate used to exclude cell aggregates and debris. Attached cells were trypsinised, ethanol-fixed, and stained with propidium iodide (PI) to indicate DNA content. Plot shows the area (vertical scale) vs. width (horizontal scale) of the PI signal.

(B) Demonstration of the positioning of the BrdU+ gate on a plot of BrdU fluorescence intensity (vertical scale) vs. DNA content (horizontal scale), by delineating a boundary immediately above the dots which appear in a no-BrdU control sample (left panel). NSLT attached, proliferating cell samples were processed in parallel. BrdU was added to the right sample for 1 hour before harvesting. Both samples were harvested, ethanol-fixed and stained with antibodies to BrdU, and propidium iodide (PI) to indicate DNA content as above.

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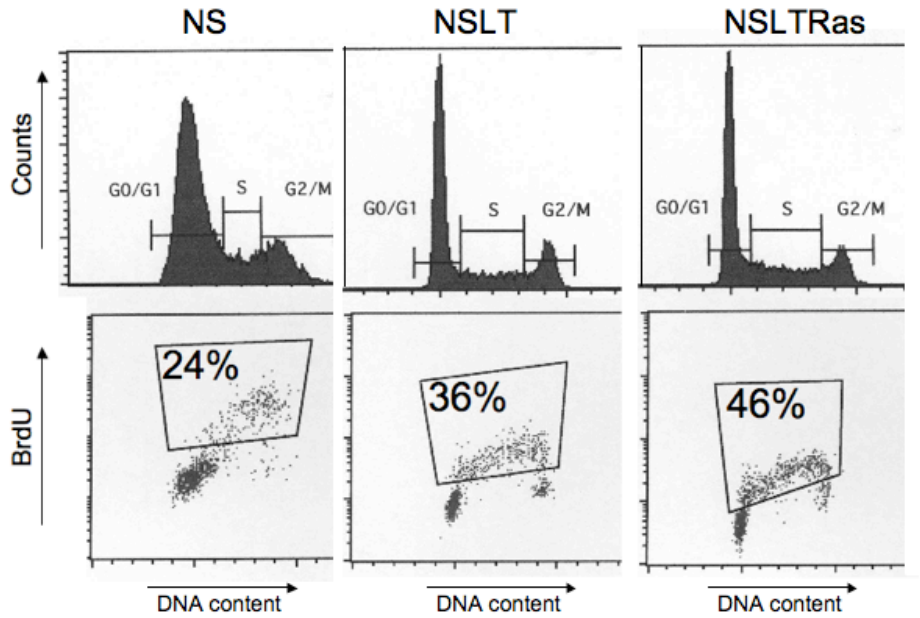

B

% Cells in phase: (Attached)	G0/G1	S	G2/M
NS	74	11	15
NSLT	62	18	20
NSLTRas	59	24	17

Figure 3- 11: Attached NS, NSLT and NSLTRas cells show normal proliferating cell cycle profiles when analysed by flow cytometry.

(A) Attached NS, NSLT and NSLTRas cells were given a 1-hour BrdU pulse directly before harvesting, fixing, and staining for flow cytometry, with antibodies to BrdU, and propidium iodide (PI) to indicate DNA content. Top panels: histograms of DNA content, with phases marked as indicated. Bottom panels: dot plots of BrdU incorporation vs. DNA content, with BrdU+ gates defined as in Figure 3- 10B. Percentages indicate BrdU+ cells for each cell type. 10,000 events were collected per sample.

(B) Quantification of the proportion of cells in each phase, as defined by markers on histograms above.

cells appearing in the G0/G1 cluster (Figure 3- 12A, left panel). Importantly, given that a proportion of cells would have been mid-cycle when put into suspension, this suggested that the majority of NS cells were able to complete the cycle and return to G1 within 24 hours even in the absence of anchorage signals.

Consistent with the increase in cell numbers, NSLTRas cells after 24 hours in suspension maintained a 'proliferating', horseshoe-shaped cell cycle profile (Figure 3- 12, right panel). The accumulation of cells in G2/M phase (55% compared to 17% in attached cells) suggests that the process of mitosis and cytokinesis may take longer in suspension. However, there was little reduction in the BrdU-positive population (Figure 3- 13A), consistent with an overall proliferation rate similar to that in attached cells, as observed in the cell counts.

In contrast, NSLT suspended cells, although no longer proliferating, did not show a profile characteristic of cell-cycle arrest. There were several points to note from the analysis (Figure 3- 12A, middle panel). Firstly, the PI profile was similar to that of the proliferating NSLTRas cells, in that cells appeared in all phases including S phase, with some accumulation in G2/M phase (Figure 3- 12B). However, in contrast to NSLTRas, the rate of BrdU incorporation in NSLT was substantially inhibited compared to the proliferating, attached cells, with only half as many cells BrdU-positive in suspension (see also Figure 3- 13A). Although BrdU incorporation in NSLT suspended cells was much reduced, it was not completely absent, as might be predicted for a non-proliferating cell sample. Finally, and importantly, many cells in S phase (i.e. with an intermediate DNA content) were clearly BrdU-negative, giving a triangular rather than horseshoe-shaped BrdU plot.

Together, these observations indicate a slower cell-cycle progression, with periods of stalling, rather than a phase-specific arrest in NSLT suspended cells. The presence of cells that show an 'S phase' DNA content between 2N and 4N, which nevertheless remain BrdU-negative, indicates that replication must have stalled for at least the duration of the one-hour BrdU pulse. To confirm the FACS data, NSLT cells were given a 5-hour BrdU pulse in suspension before retrieving, fixing within one hour, and antibody staining

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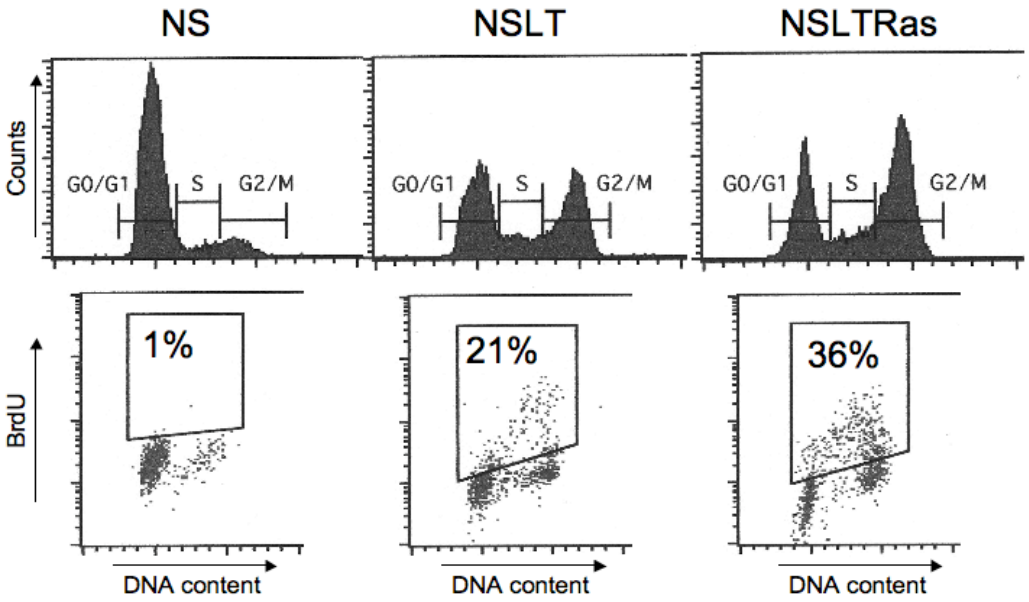

B

% Cells in phase: (Suspended 24hrs)	G0/G1	S	G2/M
NS	87	6	7
NSLT	46	13	41
NSLTRas	32	13	55

Figure 3- 12: Suspended NSLT cells show an unusual cell cycle profile.

(A) NS, NSLT, and NSLTRas cells in methylcellulose suspension were given a 1-hour BrdU pulse directly before harvesting at 24 hours, fixing, and staining for flow cytometry, with antibodies to BrdU, and propidium iodide (PI) to indicate DNA content. Top panels show histograms of DNA content. Bottom panels show dot plots of BrdU incorporation vs. DNA content, with BrdU+ gates defined as in Figure 3- 10B. Percentages indicate BrdU+ cells for each cell type. 10,000 events were collected per sample.

(B) Quantification of the proportion of cells in each phase, according to the cell cycle profiles.

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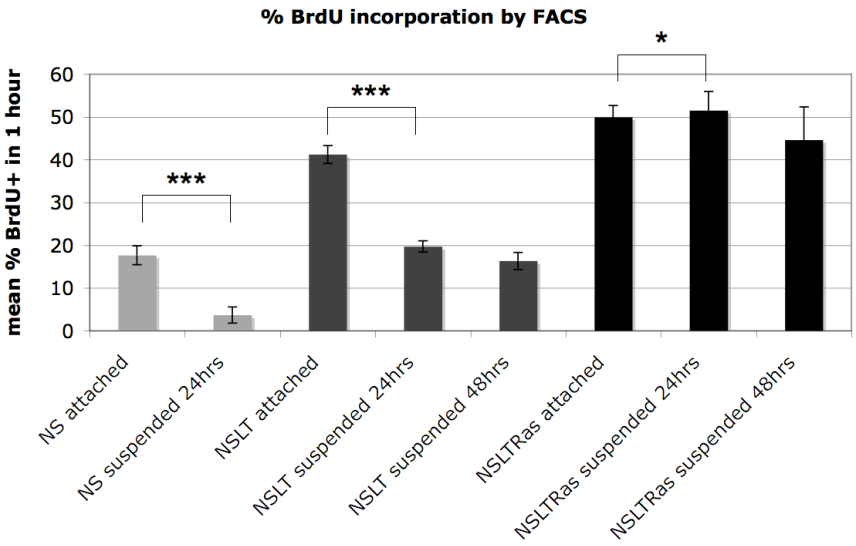

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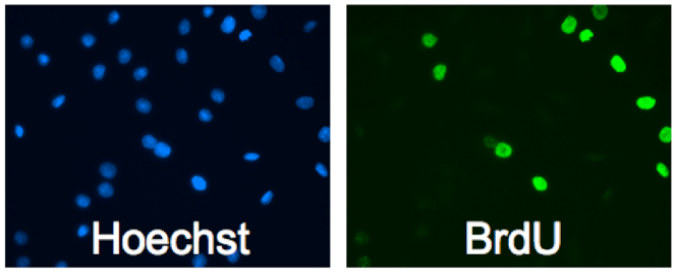

Figure 3- 13: Quantification of BrdU incorporation by flow cytometry, confirmed by immunofluorescence in NSLT cells from suspension.

(A) BrdU incorporation in NS, NSLT, and NSLTRas attached cells, and those suspended for 24 or 48 hours, following a one-hour BrdU pulse. Samples were analysed by flow cytometry and quantified by 'gating' dot plots as demonstrated in Figure 3-10B. Graph shows mean of 4 separate experiments. Error bars indicate s.e.m. * $p>0.7$, no significant difference; *** $p<0.02$, significant difference (2-tailed, unpaired t-test).

(B) Immunofluorescence staining for BrdU in NSLT cells following a five-hour pulse in 24-hour suspension. Cells were retrieved, washed to remove excess BrdU and methylcellulose and then plated on laminin and PLL-coated coverslips. Immediately after settling (20 minutes after plating), cells were fixed and immunostained to show BrdU-positive nuclei (green).

samples for immunofluorescence. Any BrdU present in the methylcellulose was thoroughly washed away before attaching the cells to coverslips. Quantification of the BrdU staining on cells after 24 hours in suspension confirmed the maintenance of low levels of BrdU incorporation in NSLT suspended cells (Figure 3- 13B).

A continued, slow rate of BrdU incorporation, coupled with an accumulation of cells in G2/M phase, raised the possibility that all NSLT cells would be able to complete S phase given enough time, and would then arrest somewhere in G2/M phase. This would place the requirement for anchorage in NSLT cells at mitotic entry or exit. The fact that the proportion of NSLT cells in G1 phase decreases from 62% to 46% when they are put into suspension suggests that cells are able to make the transition from G1 to S phase in the absence of anchorage. To see whether this trend continues, we compared FACS analysis of NSLT cells suspended for 24-hour and 48-hour time periods (Figure 3- 14A). Cells in suspension for 48 hours were given a one-hour BrdU pulse, and processed by flow cytometry as before. The PI profile and BrdU/PI plot of NSLT cells after 48 hours was similar to that of the 24 hour suspended cells. The percentages of cells in each phase are also almost identical, with little further accumulation of cells in G2/M phase, suggesting that S phase progression is extremely slow (Figure 3- 14B). The absence of any further decrease in the proportion of cells in G1 suggests that perhaps cells do not maintain their ability to enter S phase in suspension. Confirmation of whether all cells are competent to enter S phase would require samples taken after a much longer timescale, beyond the scope of this assay, since the methylcellulose medium cannot be replenished in these suspension cultures.

3.4 Chapter summary and conclusions

As documented in this first results chapter, we constructed the NS/NSLT/NSLTRas model cell system and optimised the methylcellulose method of suspension culture and retrieval, in order to investigate the novel p53- and Rb-independent anchorage checkpoint mechanism found in NSLT cells. Counts of cells retrieved from suspension supported the results from

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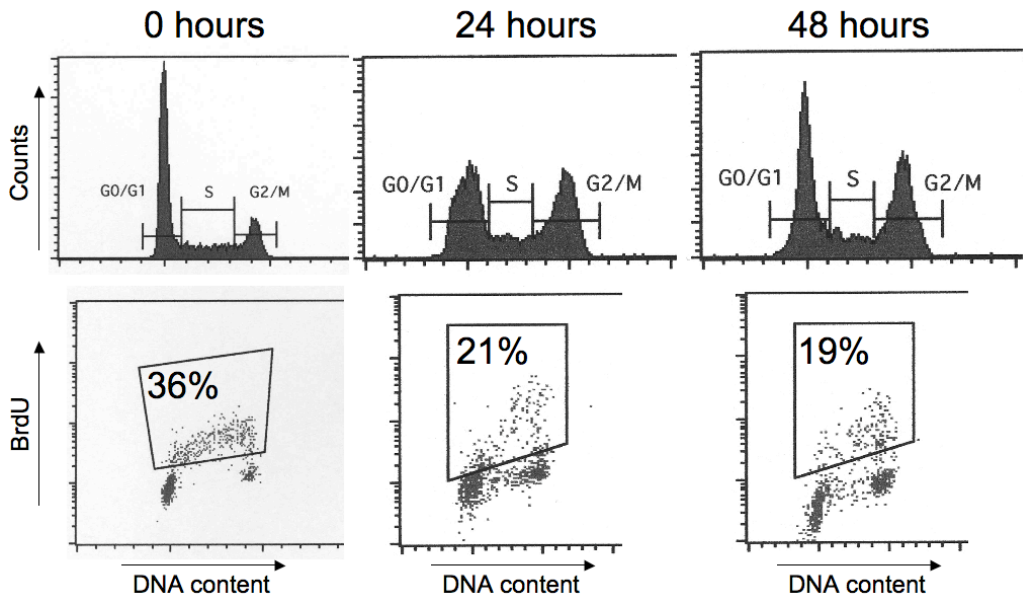

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% Cells in phase:	G0/G1	S	G2/M
NSLT (attached/ 0 hrs suspended)	62	18	20
NSLT (24 hrs suspended)	46	13	41
NSLT (48 hrs suspended)	46	12	42

Figure 3- 14: Comparison of attached and suspended NSLT cell cycle profiles after 0, 24 and 48 hours.

(A) Cell cycle profiles from attached (left) and suspended NSLT cells after 24 hours (middle) and 48 hours (right) in methylcellulose. Cells were given a one-hour BrdU pulse directly before harvesting, and stained with PI. Histograms (above) indicate frequency of DNA content in each sample; dot plots (below) show BrdU incorporation against DNA content. Percentages indicate BrdU incorporation. 10,000 events were collected per sample.

(B) Quantification of cells in each phase, according to the above histograms.

colony-forming assays in soft agar, in showing that only NSLTRas cells increased in number and were able to form colonies in suspension, while NS and NSLT cells were both strictly anchorage independent with respect to proliferation. Cells that were retrieved were able to reattach to culture dishes and resume proliferation, indicating that suspension-induced mitotic arrest was reversible in these cells, although retrieval of NSLT cells from methylcellulose was not 100% due to a minor proportion of cells that die in suspension.

NSLTRas cells showed a cell cycle profile consistent with their observed proliferation in suspension, and NS cells exhibited a classical G1 arrest, as seen in previous studies on NRK and NIH 3T3 fibroblasts (Guadagno and Assoian 1991; Zhu et al. 1996). In contrast, the NSLT cells did not show a stable arrest. Instead, flow cytometry analysis showed that suspended NSLT cells appeared in all phases of the cell cycle, and importantly, results from both FACS and fluorescence microscopy showed the maintenance of a low level of BrdU incorporation, suggesting that NSLT cells were able to progress through S phase while in suspension. This was unexpected, since the absence of any increase in the total number of cells led us to expect a complete block to cell cycle progression. DNA replication was slower in suspension, as shown by the reduced proportion of BrdU-positive cells compared to the attached sample, with periods of stalling, shown by the proportion of S-phase cells that did not incorporate any BrdU during a one-hour pulse. Analysis of cells in suspension culture also showed an accumulation of cells in G2/M phase, suggesting a secondary requirement for anchorage in G2/M phase of the cell cycle, in addition to the well-documented G1/S phase requirement seen in NS cells. This novel anchorage checkpoint is only detectable in the NSLT cells, as they require little signal input for the G1/S phase transition. The following chapter describes the investigation of the molecular mechanism inhibiting normal cell-cycle progression in suspended NSLT cells.

Chapter Four – Biochemical characterisation of model

4.1 Introduction

In the previous chapter I described the differences in cell cycle behaviour between our three model cell types: NS, NSLT, and NSLTRas, and optimised a methylcellulose suspension culture system which allows both anchorage-independent culture and maximal retrieval of cells. NSLTRas cells proliferate efficiently in suspension, while NS and NSLT cells do not increase in number at all, and either arrest in G1 or fail to complete the cell cycle, respectively. In this chapter we characterise the differences between anchorage dependent and independent cells biochemically, in particular the analysis of the composition and activity of the key drivers of the cell cycle, the cyclin-dependent kinase complexes, since the mechanism responsible for the aberrant cell cycle progression seen in NSLT suspended cells is highly likely to be the result of altered CDK regulation.

4.2 Biochemical characterisation

4.2.1 NSLT cells maintain cyclin/CDK expression in suspension

Protein samples were prepared from the three cell types: NS, NSLT and NSLTRas, from both attached and 24-hour suspended cell cultures, and the total levels of cyclins and cyclin-dependent kinases were analysed by Western blotting (Figure 4- 1). In NS cells, cyclins D, E and A and B are expressed in attached cells, but levels of cyclins D, E and A drop to barely detectable levels in suspension, indicating that anchorage signals are ordinarily required for their expression. CDK2 levels also decrease substantially in suspension, and CDKs 4 and 6 show a modest decrease, while levels of cyclin B and CDK1 in NS cells do not appear to change in suspension. It is known that both integrin and mitogenic signals are required to produce the sustained ERK activation required to induce cyclin D1 expression, in cells with an intact Rb-E2F checkpoint (Roovers et al. 1999). In the absence of cyclin D1 expression, cyclins E and A were also expressed at lower levels in NS suspended cells, since active cyclin

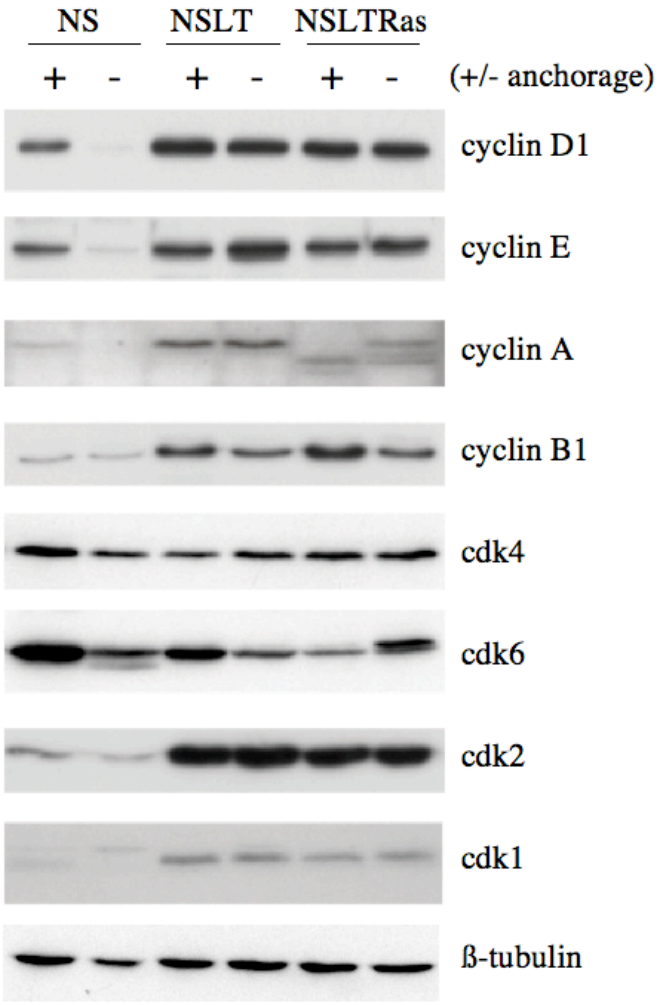

Figure 4- 1: Expression levels of cyclins and CDKs.

Western blot analysis of total lysate from attached (+) and suspended (-) NS, NSLT, and NSLTRas cells. ß-tubulin is shown as a loading control.

D-CDK4/6 is required to inactivate Rb and to promote E2F-mediated transcription (Johnson 1995).

In contrast, LT-expressing cells (both NSLT and NSLTRas) expressed higher levels of many of the cyclins and CDKs in attached cells when compared to NS. These included all four cyclins: D, E, A and B, and CDKs 1 and 2. This is consistent with the E2F-mediated derepression of the cyclin E (Ohtani et al. 1995), cyclin A (Schulze et al. 1995), CDK2 (Shiffman et al. 1996) and CDK1 (Dalton 1992) gene promoters owing to inactivation of Rb by LT, and has previously been reported in these cells (Lloyd et al. 1997). Moreover, the high expression of each of these cell-cycle regulators was maintained in suspension in both the NSLT and NSLTRas cells (compare lanes 3-4 and 5-6). This is in contrast to the NS cells, where the expression of the cyclins and CDK2 was dependent on the presence of attachment signals (compare lanes 1 and 2). The minimal expression of cyclin B1 in both NS attached and suspended cells compared to NSLT may reflect the relatively small proportion of NS cells in G2/M phase at any one time in this asynchronous cell population, given that cyclin B levels rise towards the onset of mitosis and the protein is rapidly degraded to signal mitotic exit. The higher cyclin B levels in the more rapidly proliferating NSLT and NSLTRas attached cells could be due to the higher proportion of cells in mitosis in these cultures. The presence of cyclin B in the NSLT suspended sample is a further indication that these cells are found in all phases of the cell cycle and not accumulated in arrest, in agreement with the cell cycle profiles shown in Chapter Three.

The levels of CDK4 in the three cell types were remarkably consistent, whether attached or in suspension, indicating that the levels of the CDK are not differentially regulated in the different cell types, or by cell adhesion. The related cyclin D1-associated kinase CDK6 is also detectable in all samples, and again levels do not seem to correlate either with cell type or adhesion status. The consistency of CDK4 expression in our cells is indicative of its regulation primarily by cyclin binding (Matsushime et al. 1994) and other post-translational mechanisms, rather than by control of its expression levels.

Expression of the cyclins and CDKs in NSLT and NSLTRas cells does not seem to correlate with proliferation, as the protein levels were comparable in

the anchorage-dependent NSLT and anchorage-independent NSLTRas, and expression was largely maintained in suspension in both cell types. This suggests that differences in cyclin-CDK activation may be responsible for inhibiting proliferation in the suspended NSLT cells.

4.2.2 CDK2 activity in anchorage-dependent cells is dramatically reduced in suspension

As central regulators of cell cycle progression, cyclin-CDK complexes are likely to be the eventual targets of any anchorage-dependent mechanism preventing cell proliferation in suspension. As seen in the previous section, cyclin and CDK levels were unchanged in NSLT suspended compared to attached cells, implying that anchorage-dependent regulation in these cells could be at the level of cyclin-dependent kinase activity, rather than at the level of protein induction, as in NS cells. To assess this possibility, kinase assays were carried out on immunoprecipitated cyclin complexes, to measure endogenous CDK activity associated with cyclins E, A, and B1 in attached and suspended cells. Cyclins E and A associate with CDK2, while CDK1 associates with cyclin A and cyclin B. Activity of the endogenous cyclin complexes can be measured in vitro by monitoring phosphorylation of a histone H1 substrate with radiolabelled ATP. Preliminary tests using previously frozen cell pellets showed a great deal of variability between experiments, and it was found that the consistency of results was markedly improved when unfrozen samples were used. This is most likely due to the labile nature of regulatory modifications on the cyclin-CDK complexes, which may be disrupted by freezing and thawing. Experiments were therefore designed so that fresh samples of both attached cells and 24-hour-suspended cells of all three cell types would be ready for harvesting at the same time, and lysis, immunoprecipitation and kinase assays were carried out on unfrozen cell samples on the day of harvesting. Tests using decreasing amounts of lysate in the kinase assay showed a linear relationship between protein added and the kinase activity produced, indicating that levels of substrate and ATP were not limiting.

As shown in Figure 4- 2A, cyclin E-associated kinase activity was much higher in NSLT and NSLTRas attached cells than NS, reflecting the higher

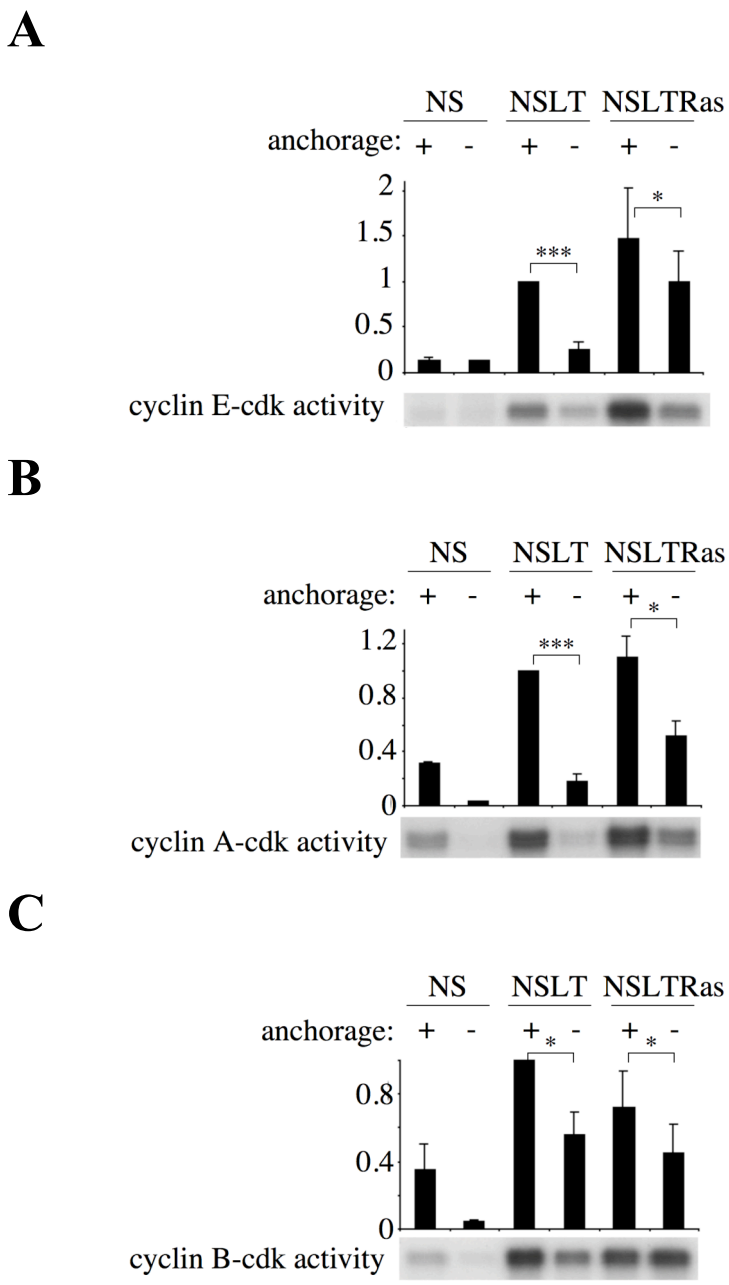

Figure 4- 2: Cyclin-dependent kinase activity in attached (+) and suspended (-) cells.

Autoradiograms show representative results for (A) cyclin E, (B) cyclin A and (C) cyclin B-associated kinase assays following cyclin immunoprecipitation from attached and suspended cell lysates. IPs were carried out using 300, 150 and 100μg of protein per sample respectively in (A), (B) and (C). Graphs show means from 4 similar experiments +/- s.e.m. * p value >0.05, no significant difference; *** p value <0.002, significant difference using a 2-tailed, one-sample t-test (suspended relative to attached).

expression of CDK2 and cyclin E as seen in Figure 4- 1. Comparison of E kinase activity in NS attached and suspended cells was difficult because phosphorylation levels were so low as to be barely above background levels. Kinase activity was highest in the NSLTRas attached cells, and did decrease somewhat in NSLTRas suspended cells. However, in NSLT cells, there was a much more pronounced decrease in CDK activity in suspension (>3 fold), with kinase activity close to basal levels. However, the reduced cyclin E-CDK activity seen in NSLT suspended cells was not less than that of the NS attached cells, which are proliferating, suggesting that other factors may be limiting NSLT proliferation in suspension.

Measurements of cyclin A-CDK activity (Figure 4- 2B) in attached cells were also consistent with the levels of cyclin A, CDK2 and CDK1 expression: much higher in LT-expressing cells than in NS cells. In NS cells, cyclin A-associated kinase activity was reduced to basal levels in suspension, also consistent with the lack of cyclin-CDK protein expression. In contrast, while levels of cyclin A, CDK1 and CDK2 protein were maintained in NSLT cells in suspension, there was a dramatic decrease in cyclin A-associated kinase activity in the suspended cells (>5 fold), to levels lower than those in proliferating attached NS cells. There was also an approximately 2-fold reduction in cyclin A-CDK activity in NSLTRas suspended cells, but in this case the kinase activity was still considerably higher than in the NS attached cells, and had little effect on cell proliferation. The relative cyclin A-CDK activity, above or below the putative 'threshold' level in NS attached cells, therefore correlates with whether cells proliferate, for both NSLT and NSLTRas samples.

Cyclin B-CDK1 activity (Figure 4- 2C) was much higher in NS attached cells compared to NS suspended cells, consistent with the lack of CDK1 expression in suspension, and the G1 arrest seen in the previous chapter. Levels of cyclin B-CDK1 activity were higher in LT-expressing cells than in NS, consistent with their higher CDK1 expression. However, the reduction in kinase activity in suspended NSLT and NSLTRas cells was less pronounced than for other cyclin-CDKs. In NS cells, cyclin B-associated kinase activity clearly correlates with cell proliferation, but in NSLT and NSLTRas cells this is not the case. This pattern of cyclin B-CDK1 activation was perhaps due to

variations in the proportion of cells in M phase, given the relatively narrow period of cyclin B-CDK activation, rather than correlation with proliferation per se (Sherr 1996).

The overall reduction in CDK activity when NSLT cells were suspended indicates that CDK activity is predominantly anchorage dependent in these cells, and that the level of inhibition of cyclin-CDK activity is likely to contribute to aberrant cell cycle progression in suspension.

4.2.3 Composition of cyclin-CDK complexes in suspension indicates increased association of p27

Since the results in Figure 4- 1 showed that total cyclin and CDK protein expression does not decrease in NSLT suspended cells, we examined the composition of the cyclin-CDK complexes further to discover how their activity was inhibited. CDK activity is regulated by at least three mechanisms: cyclin binding, activating and inhibitory phosphorylations, and the binding of small-molecule CDK inhibitors, including p21 and p27 (Morgan 1995). To analyse the mechanism responsible for inhibiting cyclin-dependent kinase activity in NSLT cells, we first determined the total levels of these CDK inhibitors in our attached and suspended cell lysates.

Western blots of total CDK inhibitor levels (Figure 4- 3A) showed that in NS cells, p27 levels were induced in suspension, as has been observed in previous studies in anchorage-dependent fibroblasts (Zhu et al. 1996; Carrano and Pagano 2001). Surprisingly, p21 levels were decreased in NS suspended cells, in contrast to previous studies suggesting that both p27 and p21 are induced following loss of cell anchorage (Fang et al. 1996; Zhu et al. 1996). In NSLT and NSLTRas cells, p21 levels were unchanged in suspension, most likely due to the LT-mediated inhibition of p53 (el-Deiry et al. 1993; Lloyd et al. 1997; Wu and Schönthal 1997). NSLTRas cells maintained barely detectable levels of p27 as well as p21, consistent with Ras-induced upregulation of Skp2-mediated degradation (Kawada et al. 1997; Bornstein et al. 2003; Bhatt et al. 2007). In contrast, p27 levels were strongly induced in NSLT cells in suspension. The expression pattern of p27 suggests a potential

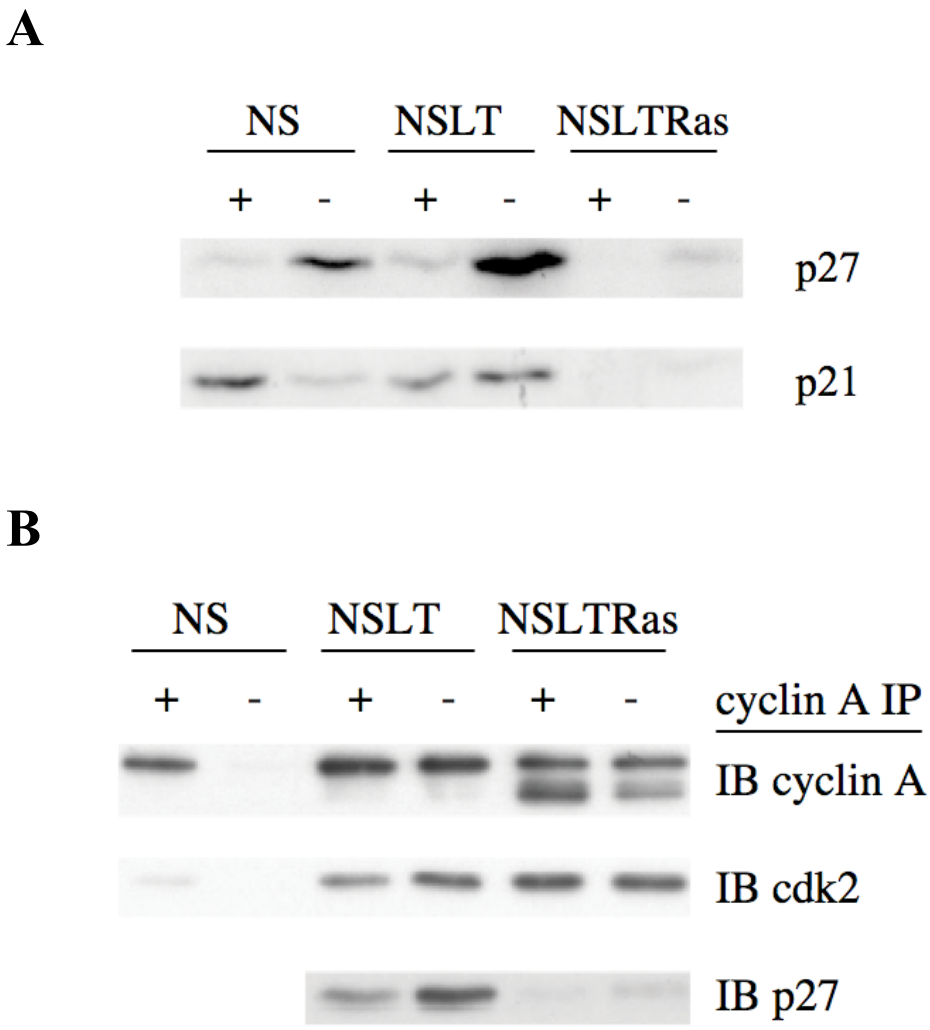

Figure 4- 3: Composition of cyclin A-CDK complexes.

(A) Total expression levels of CDK inhibitors in NS, NSLT and NSLTRas attached (+) and suspended (-) cells. 20μg of protein sample was loaded per lane.
(B) Immunoprecipitated (IP) cyclin A complexes, showing levels of cyclin A and bound CDK2 and p27 by immunoblot (IB). IP was carried out starting with 800μg of total protein for each sample.

mechanism for anchorage-dependent regulation of cyclin-CDK activity in NSLT cells.

In order to investigate the activation status of CDK complexes in attached and suspended cells, we analysed their composition, to determine levels of potentially active cyclin-bound CDK, as well as the levels of CDK inhibitors bound to the complex. In preliminary experiments, cyclin A was immunoprecipitated from cell lysates and associated proteins blotted to determine levels of cyclin-bound CDK. However, the levels of pulled-down proteins were obscured by the heavy and light chains of the IP antibody, which are present at high levels in the precipitate. To avoid this, we used cyclin A antibody chemically cross-linked to protein G beads, which successfully removed the interference of heavy and light immunoglobulin chains with the pulled-down proteins.

Blotting the separated complexes (Figure 4- 3B) showed that cyclin A and CDK2 were co-immunoprecipitated from NS attached cells, while no protein was pulled down in NS suspended cells, consistent with the lack of cyclin A in these conditions. In contrast, almost equal amounts of CDK2 was present bound to cyclin A in NSLT suspended as in attached cells. Thus, the levels of cyclin A-CDK2 complex are not responsible for the loss of kinase activity in these cells. A similarly equal amount of cyclin A-CDK2 was co-immunoprecipitated from NSLTRas attached and suspended cell lysates. Strikingly however, blotting for p27 in cyclin A complexes showed that the amount of CDK-bound inhibitor was increased in NSLT suspended cells in parallel with the increase in total p27 protein, suggesting that p27 may be responsible for the CDK inhibition seen in NSLT cells in suspension. In NSLTRas cells, no p27 was detected bound to cyclin A complexes, consistent with the low p27 levels in Ras-expressing cells presumably due to its oncogene-induced degradation (Kawada et al. 1997; Bhatt et al. 2007; Grimmler et al. 2007).

The evidence presented thus far supports a role for p27 in suspension-induced CDK inhibition and anchorage dependence in NSLT cells. To assess the importance of p27 in inhibiting CDK activity, we investigated the proportion of CDK complexes that were bound by p27 in attached and

suspended cells. To do this, NSLT attached and suspended cell lysates were subjected to p27 immunodepletion using a polyclonal anti-p27 antibody. Blotting for p27 after successive depletions (removing protein-bound beads and adding fresh beads and antibody to the sample each time) indicated that three rounds of depletion were sufficient to remove detectable p27 in the lysates (Figure 4- 4A). Non-specific 'clearing' by incubating with protein A-sepharose beads was used as a control. The immunodepletion should also remove all p27-bound protein, including any bound cyclin-CDK complexes, from the lysate. To determine the amount of unbound cyclin-CDK complex remaining, cyclin A complex was immunoprecipitated from p27-depleted and control supernatant, and blotted for CDK2 (Figure 4- 4B). Depletion of p27-bound protein had little effect on the amount of cyclin-CDK complex retrieved from attached NSLT samples, whereas it almost completely removed CDK from suspended samples, indicating that nearly all cyclin A-CDK2 complexes were p27-bound in suspension. Since p27 molecules bind and inhibit cyclin A-CDK2 with a 1:1 stoichiometry (Russo et al. 1996; Bienkiewicz et al. 2002), this strongly suggests that the binding of p27 to cyclin-CDK complexes is responsible for the inhibition of CDK2 activity in suspended cells.

4.2.4 p27 loss cooperates with LT in inducing anchorage independence

To confirm the role of p27 in preventing anchorage-independent proliferation, we took a genetic approach to knock down p27 expression in our cell system. We used a siRNA protocol that had been previously tested in the lab and used against other gene targets in Schwann cells with high knockdown efficiency. Sequences of the four designed siRNA duplexes, negative control ("scrambled") duplex and procedures used are described in Chapter 2. Briefly, siRNA was mixed with transfection reagent and added to cells overnight (17-19 hours), before replacing with fresh medium. Samples for Western blotting were harvested 24 hours after this medium change. Initial experiments to test each siRNA duplex on NSLT attached cells, alone and in combination, at concentrations between 0.5 and 2nM, gave little if any knockdown as judged by Western blotting (Figure 4- 5A). Different mixtures of two siRNAs, applied to

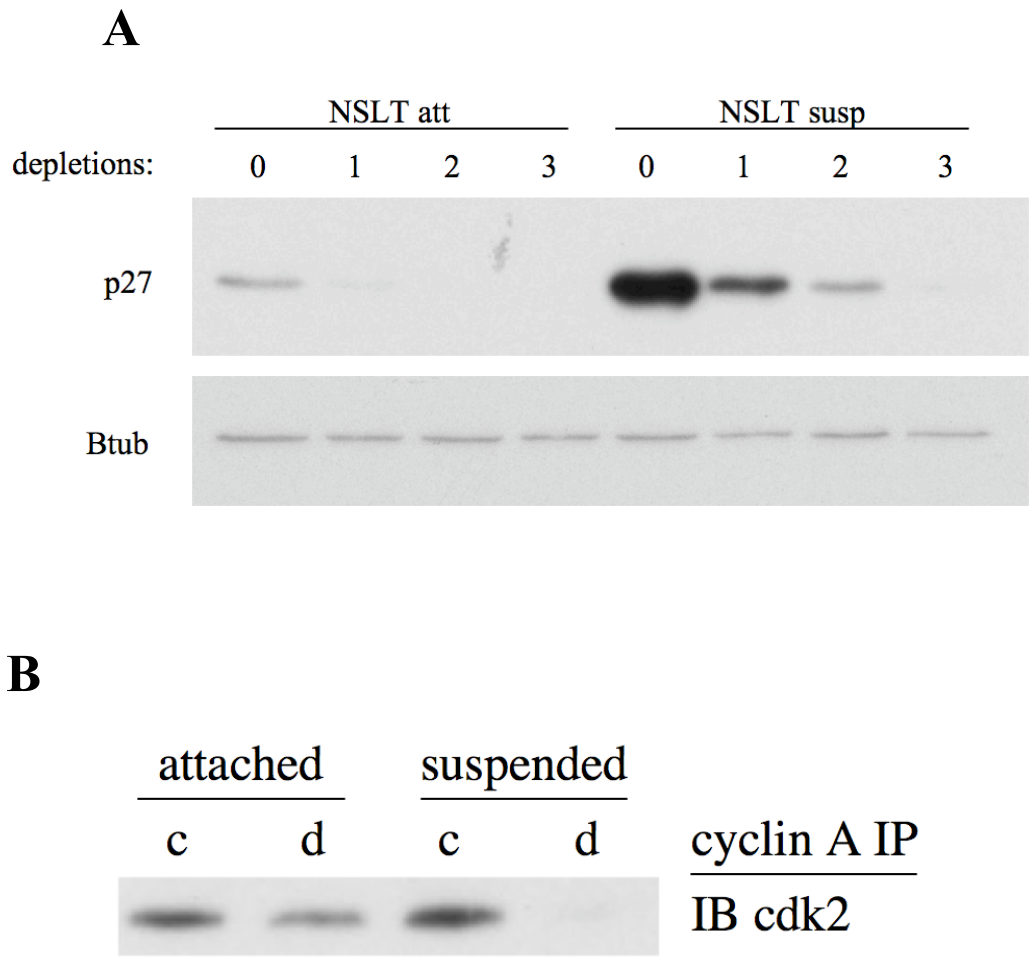

Figure 4- 4: Depletion of p27-bound complex shows most cyclin A-CDK2 complex is inhibited by p27 in suspension.

(A) p27 depletion of NSLT lysates, showing p27 levels initially (0) and after 1, 2 and 3 rounds of p27 immunodepletion (1-3) in samples from attached (att) and suspended (susp) cells. β–tubulin (Btub) is shown as a loading control.
(B) Immunoblot (IB) shows uninhibited CDK2 remaining in control (c) and p27-depleted (d) lysates, by cyclin A immunoprecipitation (IP). IP was carried out starting with 250μg of total protein for each sample.

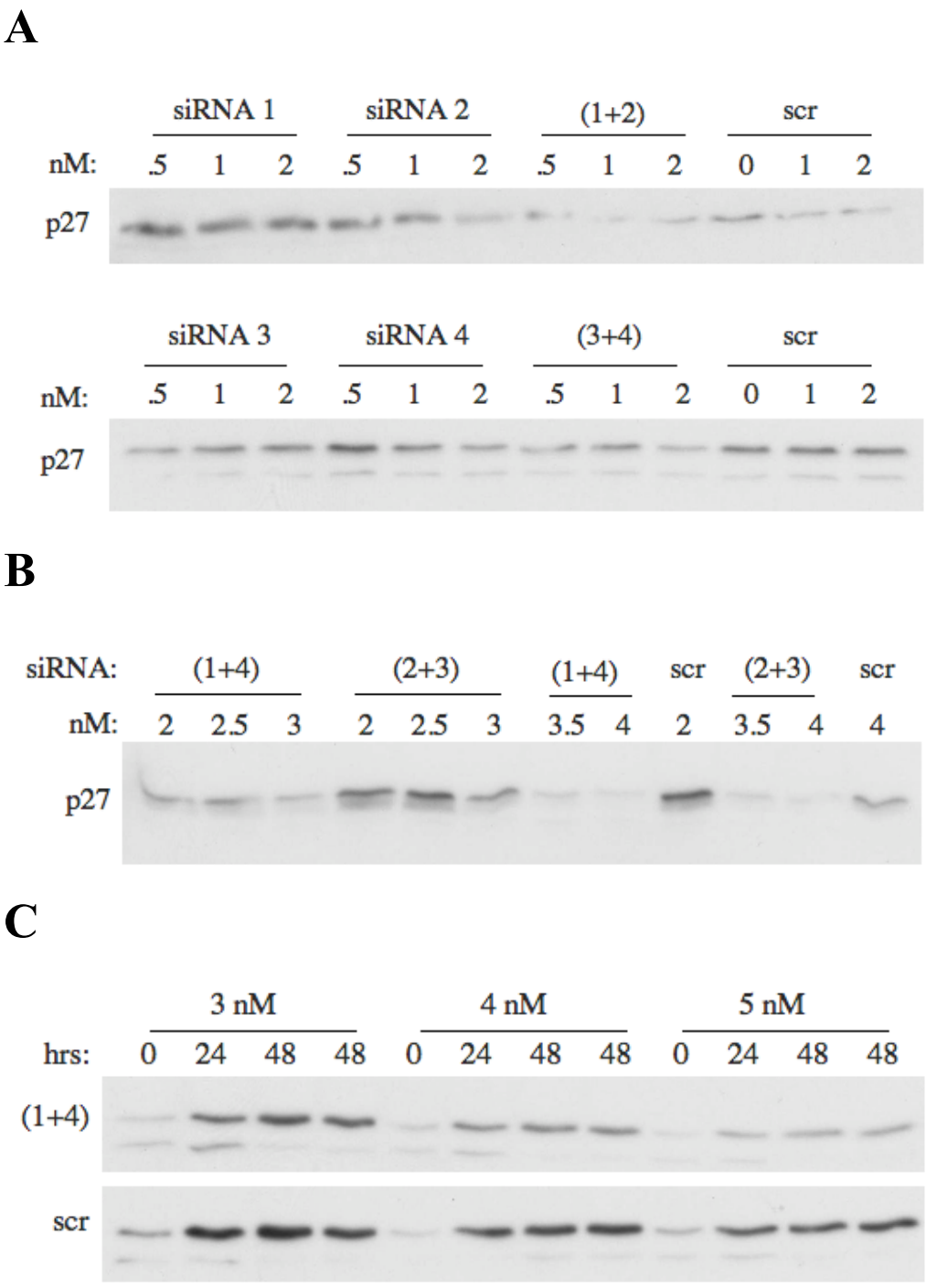

Figure 4- 5: Testing of p27 siRNA.

(A), (B) and (C): Cells were transfected with the indicated duplex(es), 1, 2, 3, 4, or negative control siRNA, 'scr', overnight at various nanomolar (nM) concentrations, and harvested the indicated number of hours (hrs) after medium change the following morning. If no time is given, samples were harvested at 24 hours post-medium change. (A) and (C) blots used NSLT cells, (B) is of NS cell samples. All blots indicate total p27 levels, and each lane represents the protein from one well of a 6-well plate.

NS cells at higher concentrations up to 4nM produced a more efficient knockdown (Figure 4- 5B). The (1+4) combination was subsequently tested on NSLT cells at 3-5nM and cell samples were harvested over a time course of 48 hours (Figure 4- 5C). The 'time zero' samples all had very low levels of p27, which was attributed to the medium change immediately before their harvesting. Knockdown was most efficient at 5nM (compare right-hand lanes on (1+4) with those on the 'scr' blot), although treatment of cells with control siRNA at this level also had a small reducing effect on p27 levels. There was little difference between the knockdown efficiencies at 24 and 48 hours, meaning this protocol could be used in time course experiments up to two days in suspension.

The optimised siRNA procedure, using a mixture of duplexes 1 and 4 at a combined concentration of 5nM, was then scaled up for use on NSLT cells both attached and in suspension. As before, attached, proliferating cells were transfected with siRNA overnight, followed by replacement with fresh medium. After 6 hours, half the cells were transferred to methylcellulose suspension culture, and 24 hours post-medium change, both attached and suspended cells were harvested. Western blotting of the total cell lysates shows efficient knockdown in attached cells, consistent with the preliminary data, but a weaker knockdown effect in suspended cells (Figure 4- 6A, top blot). Cyclin A was also immunoprecipitated from samples of the lysate to determine the levels of p27 specifically bound to cyclin-CDK complexes. Here the results showed a moderate reduction in bound p27 in attached cells, but little change in cyclin-bound p27 in suspended cells (Figure 4- 6A, lower blot). Cell counts from control and target siRNA-transfected suspension cultures were similar over 2 days, consistent with the failure to deplete p27 efficiently (Figure 4- 6B). Perhaps the stabilisation of p27 protein following loss of anchorage was able to increase p27 levels in suspension even after knockdown, or the knockdown did not last long enough for levels to remain suppressed for 24 hours in suspension. It is also possible that the increased binding of p27 to cyclin A complexes in suspension protected it from degradation, further undermining the effect of the knockdown on p27 function.

Given the initial success of the siRNA approach in reducing p27 levels in attached cells, it was hypothesised that a more long-lasting knockdown might

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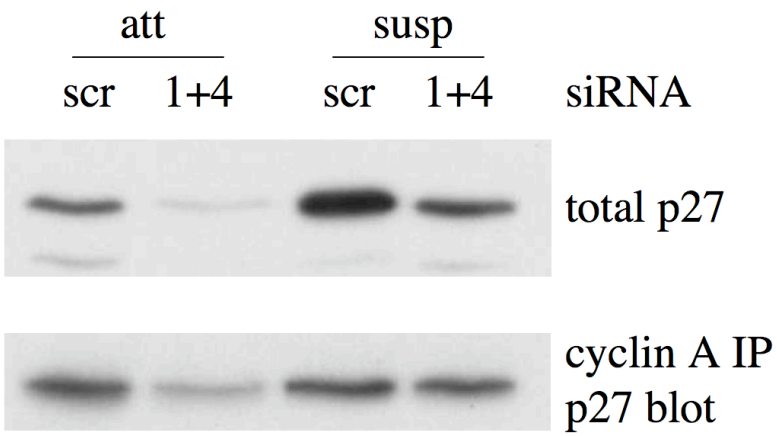

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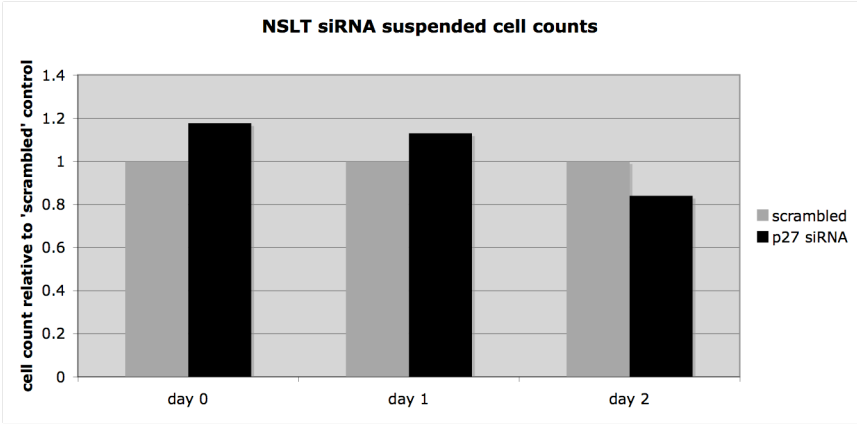

Figure 4- 6: p27 siRNA in attached and suspended cells

(A) p27 siRNA knockdown in NSLT attached (att) and suspended (susp) cells. Attached cells were transfected with negative control (scr) or p27-targeted (1+4) duplexes at 5nM concentration. Suspended cells were seeded into methylcellulose 6 hours post-medium change; all cells were harvested at 24 hours. Upper blot shows total p27; 25µg protein was loaded per lane. Lower blot shows cyclin A-bound p27; cyclin A immunoprecipitation was carried out using 300µg of protein per sample. (B) Cell counts from siRNA-treated cells seeded into methylcellulose at medium change, and retrieved immediately (day 0) or after 1-2 days.

allow time for complete elimination of p27 in attached cells before transferring them to suspension, thereby overcoming the problem of protein stabilisation and preventing p27 accumulation in suspended cells. To effect stable knockdown, short hairpin RNA constructs (shRNAs) targeting the p27 sequence were cloned into pSiren-RetroQ-zsGreen (see Materials and Methods). This retroviral vector plasmid should allow both constitutive expression of the shRNA construct, and identification of the knockdown cells via the coexpression of a green fluorescent tag. Three different shRNAs were designed using Clontech's 'RNAi target sequence selector' and 'shRNA sequence designer' online tools, targeting three different regions of the rat p27 gene at sequences unique to the rat genome (confirmed by BLAST searches). A fourth shRNA sequence was taken from the literature (Itoh et al. 2007), having been successfully used to target p27 in mouse cells (at a target sequence conserved between mouse and rat) using a similar vector. Once ligated into pSiren-RetroQ-zsGreen, the four shRNA vectors were transformed into bacteria and isolated colonies were grown up, from which plasmid DNA was purified. The presence of an insert was confirmed in all four cases by restriction digest at an Mlu1 site unique to the shRNA sequences (Figure 4- 7A). After expansion of the constructs in bacteria and extraction of plasmid DNA by maxi prep, the identity of each insert was verified by sequencing. The U6 promoter sequence, located approximately 50bp upstream of the insert site, was used as a forward sequencing primer, as recommended by the vector manufacturer. Plasmids with insert targets "2", "3" and "itoh" were 100% matched to their correct insert sequence, while insert target "7" was sequenced correctly for the first 18 nucleotides before the read failed, probably caused by the confounding secondary structure of the hairpin.

Each construct was transfected into the Phoenix retroviral packaging cell line and the virus used to infect populations of NSLT cells. Following retroviral infection, cells were sorted by flow cytometry to purify the most highly green-fluorescent cells expressing each shRNA vector (Figure 4- 7B). p27 expression was assessed in the sorted cell lines by Western blotting of both attached and suspended cells, and cells were also seeded into soft agar suspension to assay colony formation. Western blots of both attached and suspended shRNA cells gave puzzling results. In attached samples (Figure 4- 8A), cells infected with

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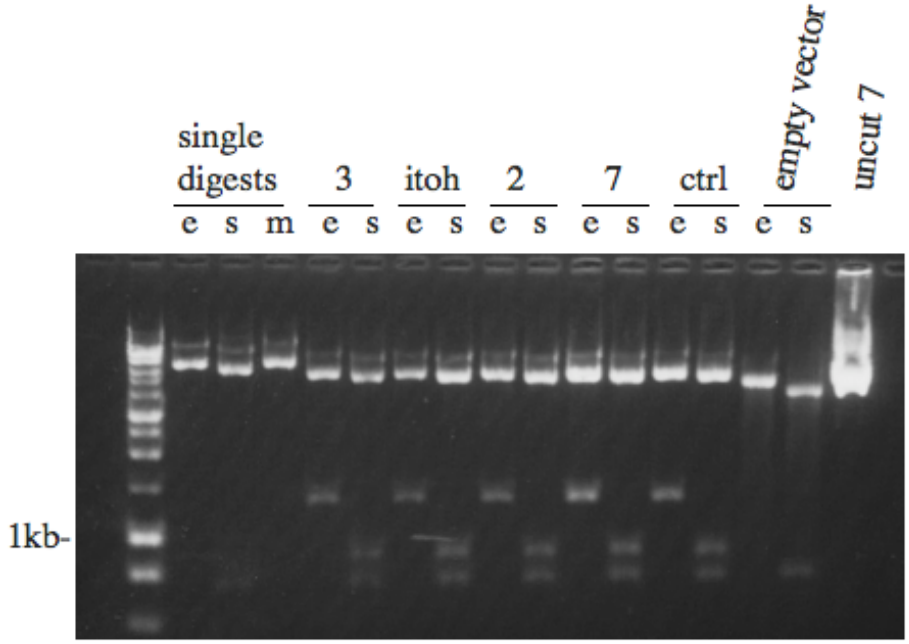

B

Cell sorting by FACS	No. of zsGreen cells retrieved	% zsGreen
LT p27 shRNA 2	723000	11%
LT p27 shRNA 3	412400	16%
LT p27 shRNA 7	673100	21%
LT p27 shRNA "itoh"	1231000	16%
LT shRNA neg. control	646700	28%

Figure 4- 7: Verification of shRNA constructs and cell sorting

(A) Restriction digests of shRNA constructs. All are double digests with Mlu1 except for the controls indicated. 'e'= EcoRV, 's'= Sal1, 'm'= Mlu1. All give the expected band pattern for vectors containing an Mlu1 insert (see Materials and Methods).
(B) Table showing percentage of cells obtained after sorting for the highest-fluorescing green cells expressing each construct.

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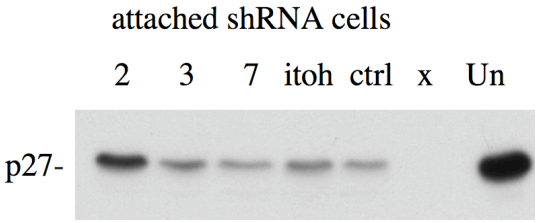

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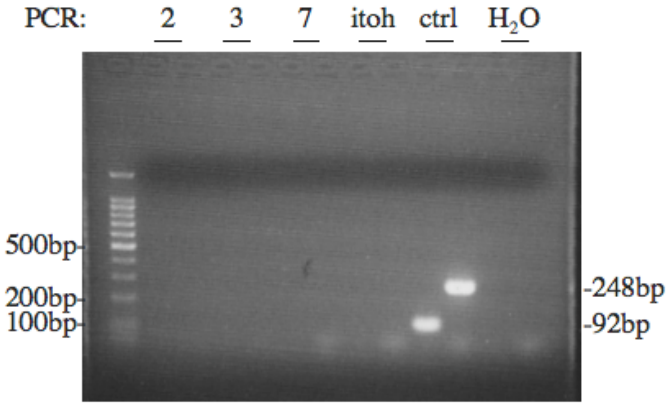

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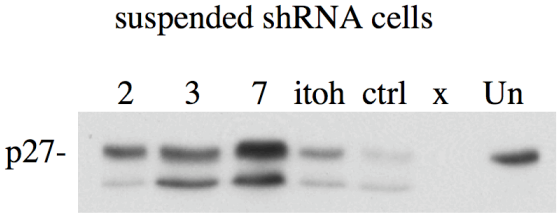

Figure 4- 8: p27 knockdown in purified shRNA-expressing NSLT cells.

(A) p27 protein in attached shRNA cells by Western. '2', '3', '7' and 'itoh' are the four shRNA constructs. ctrl= identical vector containing a 'half-hairpin'. X=blank lane. Un= uninfected control (NSLT suspended cells). 30µg protein was loaded per lane.
(B) PCR products from genomic PCR using primers specific to the negative control (ctrl) insert. Only genomic DNA from the control cells produces PCR product. See Materials and Methods for primer positions.
(C) p27 protein in suspended shRNA cells by Western. 7.5µg protein was loaded per lane. Labels as above.

the control vector express the lowest levels of p27, with shRNA populations '3', '7' and 'itoh' expressing similar low levels, and sample '2' showing the highest p27 levels. This was so unexpected that we wanted to verify that the control and target shRNA cell populations were expressing the correct construct. Genomic DNA was purified from the sorted cells, and PCR was performed, using primers we designed to detect the presence of the control shRNA insert in the pSiren-RetroQ-zsGreen vector (see Materials and Methods). The reverse primer was obligatorily targeted to the negative control insert sequence, and two alternative forward primers were used in the PCR reaction. Running the PCR products on a gel (Figure 4- 8B) demonstrated that the cell lines did contain the expected constructs. We seeded each shRNA cell population into methylcellulose suspension, and analysed p27 expression and cell number in the suspended cells. p27 knockdown did not reduce protein levels in any of the shRNA-expressing cells more than in those expressing the control vector, and in some cases p27 levels were higher than in the uninfected NSLT control (Figure 4- 8C). The pattern of p27 expression between the four cell pools also differed from that in the attached cells: here, the 'itoh' sample had the lowest p27 levels, and '7' the highest. Cells expressing the p27-targeted shRNA constructs were seeded into soft agar suspension, and no colonies were observed after 5 weeks. At this point, the shRNA approach was abandoned, reasoning that RNA interference may not be appropriate for use in situations where post-translational regulation is so important in determining the levels of the target protein.

An alternative, genetic approach to assess the role of p27 in anchorage dependent proliferation was to use cells from a germline p27-deficient mouse. Instead of using cells from p27-null mice, we obtained frozen MEFs from mice whose p27 gene had been disrupted by targeted insertion of a *neo* cassette, thereby truncating the protein. This mutant p27 is missing the binding site for CDKs, and results in a protein unable to inhibit cyclin-CDK complexes (Kiyokawa et al. 1996). Use of this p27 mutant would therefore clarify whether it is specifically the CDK inhibition activity of p27 that is necessary for preventing anchorage-independent proliferation, rather than any other property of the protein. Mice expressing only this "p27Δ51" truncation mutant exhibit

hyperplasia, and an enlarged body phenotype, that resembles the p27-null mouse (Fero et al. 1996; Nakayama et al. 1996).

Since MEFs are extremely prone to culture-induced senescence and spontaneous transformation, we cultured both p27Δ51 and equivalent wild-type MEFs in physiological (3%) oxygen conditions in which they have been shown not to senesce (Parrinello et al. 2003). We also used them at passage 1, to minimise the effect of culture shock on the cells, and the chance of unscheduled mutation. Retroviral infection of both cell types was performed one day after thawing the cells at passage 1, with either pBabe-puro empty vector or pBabe-puro containing Large T (see Materials and Methods). Immediately following infection, without subjecting the cells to potentially damaging drug selection, cells were seeded into soft agar at 4000 cells per well. Both wild type and p27Δ51 cells remained strictly anchorage dependent after infection with pBabe-puro, with less than one colony identified in these cultures on average (Figure 4-9). Most wild-type MEFs expressing LT also remained anchorage dependent (<1% colony formation), but those lacking functional p27 formed colonies in soft agar at more than twice this rate, with over 8 colonies visible on average in each field of view. Considering that the rate of LT infection in these cells may be low due to the lack of drug selection, the cooperation of p27 loss with LT in inducing colony formation is striking. The loss of anchorage dependence in LT-expressing p27 mutant cells, in combination with the measurements of p27 induction and CDK inhibition described above, was strong evidence that p27 is required to prevent anchorage-independent proliferation in cells lacking functional p53 and Rb checkpoints.

4.3 Chapter summary and conclusions

In this chapter we have investigated the mechanism mediating anchorage dependence in NSLT cells, and have shown that, while the cyclin/CDK expression profile of these cells is similar to that of the anchorage-independent NSLTRas, the activity of the CDKs is substantially reduced in suspension. Both cyclin E and A-associated kinases show a significant reduction in activity in NSLT suspended cells. This differs from the inhibition of cyclin E-dependent kinase activity and lack of cyclin A expression previously observed

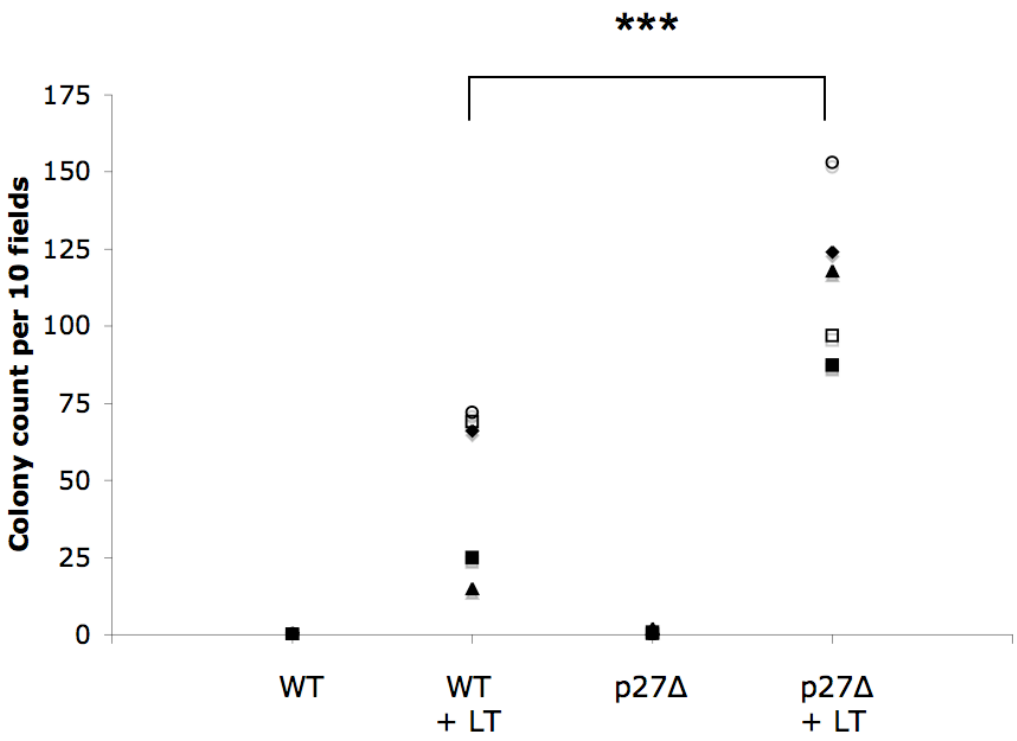

Figure 4- 9: Colony formation in wild type (WT) and p27-deficient (p27-) MEFs.

Soft agar assay of wild type (WT) and p27-deficient (p27Δ) MEFs, expressing either SV40LT (+LT) or empty vector. p27Δ MEFs express a truncation mutant (p27Δ51) unable to bind and inhibit CDKs. Cells were seeded in soft agar and colonies counted after a week in 10 microscope fields per well and at least 2 wells per cell type. Graph shows counts from 5 similar experiments. *** $p<0.005$, significant difference (2-tailed, unpaired t-test).

in human and rat fibroblasts due to E2F repression in suspension (Guadagno et al. 1993; Carstens et al. 1996; Fang et al. 1996; Schulze et al. 1996). In NSLT cells, cyclin levels were unchanged in suspension, and inhibition was at the level of CDK activation.

It is clear that the reduction in cyclin-CDK activity in NSLT cells correlates with a strong upregulation of the CDK inhibitor p27, making p27 a good candidate for the inhibition of NSLT cell proliferation in suspension. This hypothesis is supported by the results in this chapter, showing that the majority of CDK2 is p27-bound in NSLT suspended cells. Expression of LT in cells lacking functional p27 results in colony formation, indicating that loss of p27 is able to cooperate with LT in inducing anchorage independence. p27 is therefore responsible for inhibiting cell proliferation in checkpoint-deficient cells following loss of substrate adhesion.

Chapter Five –Genomic instability

5.1 Introduction

In the previous chapter I identified the mechanism by which NSLT cells fail to proliferate in suspension. I showed that anchorage dependence in checkpoint-deficient cells (lacking p53 and Rb activity) is the result of CDK inhibition by p27, and that loss of this inhibition results in anchorage-independent proliferation, as measured by colony formation in soft agar (Figure 4-9). However, the results presented in Chapter 3 showed that suspension-induced arrest in NSLT cells is only partial, in that proliferation is inhibited but DNA replication continues, albeit slowly, in the absence of anchorage. In this chapter, we analyse the effects of this aberrant cell cycle progression on the genomic stability of these cells, and the functional consequences for their tumourigenic potential.

5.2 NSLT suspended cells develop >4N DNA content

In initial experiments, we looked for evidence of rereplication in suspension, generating cells with a DNA content greater than 4N. The FACS data for cell cycle profiles shown in Chapter 3 used a 'gate' on the PI channel of the flow cytometer, in order to analyse only single cells with a DNA content between 2N and 4N, thus excluding both debris and cell aggregates in the sample. This gate (R1) is defined on a dot plot of area versus width of the PI signal (FL2-A vs. FL2-W), as shown in Figure 5- 1A. Gates R8 and R9 were not used to exclude cells from analysis, but are indicated to show the likely location of debris and cell clumps respectively. Single cells with a DNA content greater than 4N would appear in the top left corner of the plot, so we re-examined flow cytometric profiles of attached NS and NSLT cells by extending the R1 gate to detect these (Figure 5- 1A).

Quantification of the percentage of cells with >4N DNA content revealed firstly that neither the NS nor NSLT attached cells had a significant >4N DNA population (Figure 5- 1), indicating that LT expression did not in itself cause genomic instability in these cells, despite the inactivation of Rb and p53. This may seem surprising, as p53 and Rb have previously been found to

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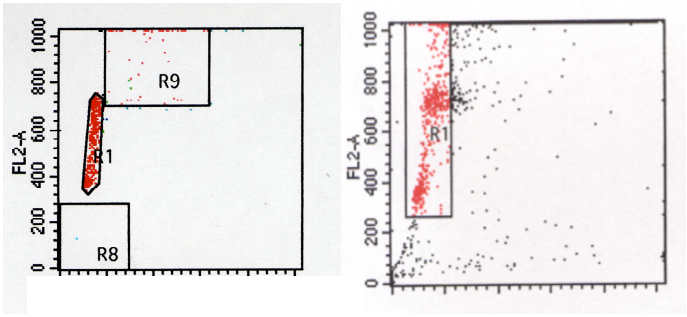

Attached cells Suspended cells

B

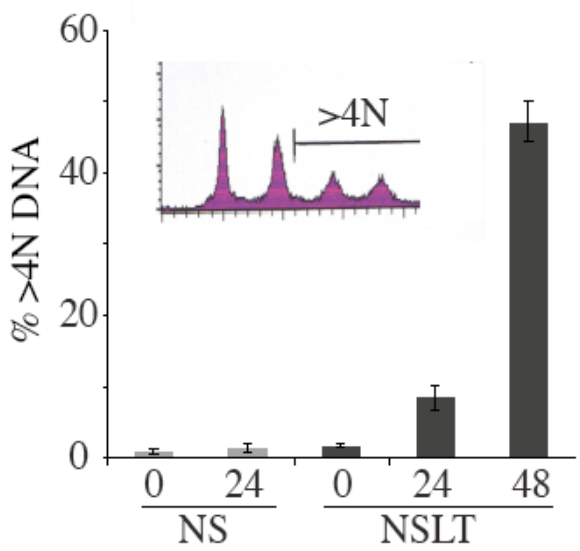

Figure 5- 1: NSLT cells over-replicate in suspension.

(A) Left: Example dot plot from attached, proliferating cell sample stained with PI. Vertical scale indicates increasing DNA content; horizontal scale indicates increasing width of signal, allowing doublet discrimination. R1 'gate' delineates data included in initial cell cycle profile; R8 shows position of cell debris (below 2N DNA content); R9 shows position of cell aggregates (above 4N DNA content, but signal width indicates more than one cell). Right: Example dot plot from suspended NSLT cell sample stained with PI. Vertical scale indicates increasing DNA content; horizontal scale indicates increasing width of signal, as before. The R1 'gate' has been extended upwards to define data included for the >4N DNA profile: this gate was used to draw a

histogram for each sample like the one in (B), from which the % >4N DNA was calculated.

(B) Quantification of >4N DNA content from FACS profiles of attached (0hrs) and suspended (24/48hrs) cells. Cells were fixed and stained with PI to indicate DNA content, and the percentage of >4N DNA cells was quantified on histograms as shown, using the extended R1 gate defined in (A) to exclude cell aggregates and debris. Graph shows the mean of at least three samples, each consisting of at least 10,000 cell events. Inset is the extended PI profile from NSLT cells after 48 hours in suspension.

prevent rereplication and double-strand break accumulation (Almasan et al. 1995; Vaziri et al. 2003; Pickering and Kowalik 2006; Srinivasan et al. 2007), so their combined loss might be expected to lead to genomic instability. However, the culture conditions used for our Schwann cells are likely to contribute to their stability: the use of minimal amounts of serum in cell medium (3%) prevents mitogen over-stimulation and replication stress leading to DNA damage (Loo et al. 1987; Mathon et al. 2001; Fikaris et al. 2006). The importance of the correct extracellular conditions for maintaining genomic stability is exemplified by the normal development of p53-deficient mice, demonstrating relatively low levels of genetic instability in cells (Donehower et al. 1992), in contrast to the highly unstable genomes of p53-deficient mouse embryo fibroblasts in culture (Harvey et al. 1993).

NS suspended cells rarely appeared with a DNA content over 4N, consistent with their tight G1 arrest. In contrast, the NSLT suspended cells showed a cell cycle profile with 4 clear peaks, indicating a substantial degree of over-replication (Figure 5- 1). Almost 50% of cells had a DNA content greater than 4N after 48 hours, suggesting that the majority of NSLT cells overreplicate when placed into suspension. The gradual emergence of this profile was likely due to the general slowing of cell cycle progression, indicated by the low levels of BrdU incorporation seen in suspended cells (Figure 3-12).

The observation that the >4N DNA profile appeared as two discrete peaks and not a continuous spread would indicate that in the majority of cells, DNA replication origins were re-licensed and all re-fired to initiate a second S phase without an intervening mitosis (endoreduplication). Uncoordinated re-firing of replication origins (re-replication) would instead result in cells with a continuous range of DNA contents, rather than a multiple of the whole genome (Porter 2008). The possibility of origin re-licensing in suspension will be discussed in Chapter Six.

5.3 Metaphase spreads show genomic instability in NSLT suspended as well as NSLTRas cells

To further investigate the over-replication phenotype seen in suspension, metaphase spreads were prepared from both attached cells and those recovered from methylcellulose culture. Recovered cells from suspension were plated straight into the colchicine-related microtubule poison Demecolcine for two hours (see Materials and Methods), thus allowing them to enter mitosis while attached, but blocking them in metaphase. This treatment selects for those cells that rapidly enter mitosis when replated.

The chromosome counts for NSLT cells were striking (Figure 5- 2). While attached cells, and those which had been in suspension for 24 hours, predominantly had a normal diploid chromosome number (42 in rat cells), NSLT cells in suspension for 48 hours showed a significant minority of nuclei with more than the usual number of chromosomes (10%; median chromosome number 78.5). In agreement with the DNA profiles, these nuclei most commonly contained about double the usual number of chromosomes, suggesting an 8N DNA content. However, there were also nuclei that contained an aneuploid number of chromosomes between diploidy and tetraploidy. This suggested that perhaps endoreduplication cycles did not occur in all chromosomes, or that chromosomes had been lost or fused. Aneuploidy has been reported to arise via a tetraploid intermediate, notably in p53-null cells (Fujiwara et al. 2005; Ganem et al. 2007), suggesting that intermediate chromosome numbers could have emerged from tetraploid cells that had then lost chromosomes. The technique of chromosome painting could clarify these intermediate karyotypes, by identifying the exact complement of chromosomes together with any breakages or fusions (Buwe et al. 2003).

NS attached cells had a stable diploid complement of chromosomes (Figure 5- 2A, left), and the protocol employed for preparing metaphase spreads did not produce any from NS suspended cells, presumably because G1-arrested cells did not have time to reach metaphase following their retrieval from methylcellulose. Since the rate of BrdU incorporation in suspended NS cells was essentially zero, their chromosome complement would not be expected to differ from the diploid attached cells. NSLTRas cells, meanwhile, had a

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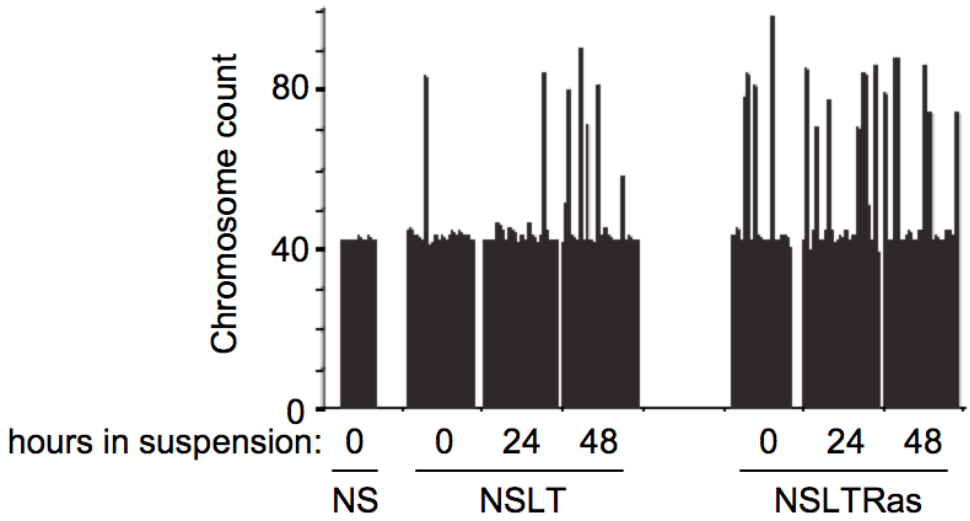

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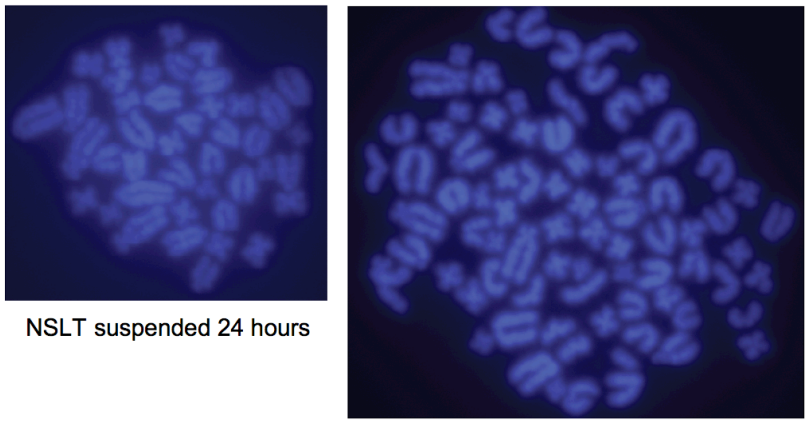


Figure 5- 2: NSLT suspended cells develop both aneuploid and tetraploid nuclei.

(A) Chromosome counts from metaphase spreads of attached (0) and suspended (24, 48) NS, NSLT and NSLTRas cells. Each vertical black bar represents one spread count; between 23 and 52 spreads were counted per sample. Diploid chromosome complement is 42.

(B) Fluorescence micrographs show example diploid (left) and tetraploid (right) metaphase spreads, from NSLT cells after 24 and 48 hours in suspension respectively. DNA is stained with DAPI.

variable chromosome number even when attached, consistent with the known cooperation between p53 loss and Ras activation in inducing genomic instability (Hundley et al. 1997). This inherent instability did not change in suspension, unlike the NSLT cells, whose unstable chromosome number only developed following loss of anchorage (Figure 5- 2).

The relatively low percentage of cells with extra chromosomes detected in NSLT samples compared to those with a >4N DNA content may be due to the metaphase spread protocol, using cells recently retrieved from suspension. The method used is very selective for cells which (a) survive the methylcellulose retrieval process, (b) are able to reattach quickly in the presence of a drug, and (c) enter mitosis and reach metaphase within two hours of reattachment. The second two points in particular would be expected to seriously limit the number of metaphase spreads produced from cells which had undergone aberrant DNA replication in suspension, which could explain the discrepancy between the metaphase spread and FACS data estimates of abnormal DNA content. It is unlikely that adding demecolcine to cells in methylcellulose would be more representative, as unless they actually entered (abortive) mitosis in suspension then spreads of metaphase chromosomes could not be made, and it would be difficult to ensure that cells received the drug in appropriate concentrations within the gel. The short period of replating allowed cells that had undergone DNA replication in suspension to reach metaphase, but the presence of the microtubule-depolymerising drug demecolcine prevented cells dividing while attached, meaning that this reattachment should not alter the chromosome complement of retrieved cells.

5.4 Giant nuclei only appear in NSLT suspended cells

To clarify whether the tetraploid cells detected by our experiments had arisen through failure to enter or complete mitosis (resulting in a single ‘giant’ nucleus), or from cytokinesis failure following a successful mitosis (resulting in a binucleate cell), attached and suspended cells were stained with CellTracker™ cytoplasmic dye, fixed on coverslips, and stained with Hoechst to visualise the nuclei. Suspended cells were allowed to reattach only during the CellTracker™

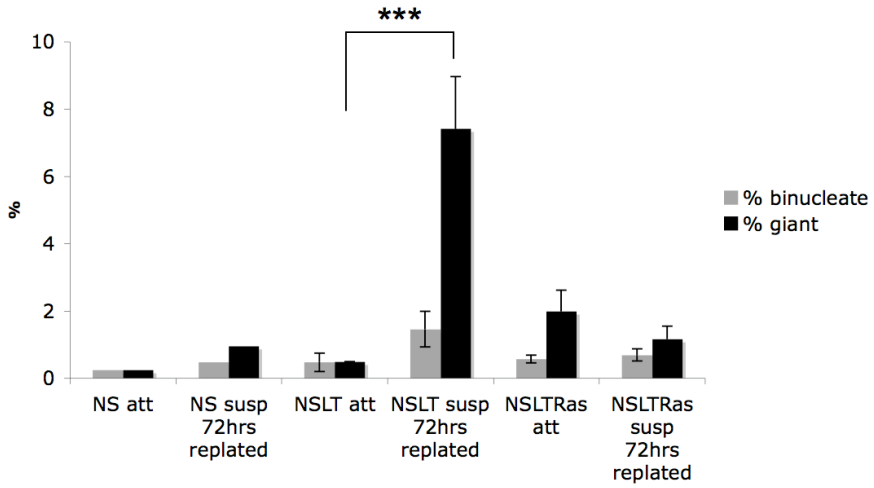
treatment, for a total of one hour, to minimise any effect attachment could have on nuclear size. In agreement with the previous results, NS and NSLT attached samples, as well as NS replated, showed neither binucleate cells nor giant nuclei, consistent with their stable genotype (Figure 5- 3A). In contrast, the NSLT cells replated from suspension had 10% abnormal nuclei. The vast majority of abnormal cells had giant nuclei rather than being binucleate. This suggested either that cells were unable to enter mitosis, and eventually re-entered S phase, or that there was a failure to complete mitosis, resulting in re-fusion of the daughter nuclei. Aborted cytokineses can also result in fusion of the newly formed nuclei, as well as formation of binucleate cells (Fujiwara et al. 2005). The proportion of abnormal nuclei detected was consistent with the metaphase-spread data. In agreement with this, the number of abnormal nuclei only increased in suspended NSLT cells. This reaffirms our observation that loss of p53 and Rb checkpoints alone does not result in genomic instability, but that loss of anchorage precipitates its development in these checkpoint-deficient cells.

Surprisingly, NSLTRas cells did not have a high percentage of abnormal nuclei in this assay even though the chromosome numbers were so variable. This could be due to the distinct morphology of NSLTRas cells compared to NS and NSLT (see Figure 3-1), making comparisons between normal and abnormal nuclei difficult. However, it is clear that in contrast to NSLT cells, and in agreement with the metaphase spread results, the proportion of abnormal NSLTRas cells did not increase in suspension. This underlines the anchorage independence of the NSLTRas cell cycle.

5.5 CDK inhibition in attached cells can reproduce the suspended cell phenotype

Our results indicate that the failure of proliferation seen in NSLT suspended cells is due to CDK inhibition (Chapter Four). To determine whether the inhibition of CDK activity in suspension was sufficient to cause the observed loss of anchorage-induced over-replication and instability in NSLT cells, we examined whether inhibiting CDK activity in attached cells could induce genomic instability. We used the CDK inhibitor Roscovitine, which

A



B

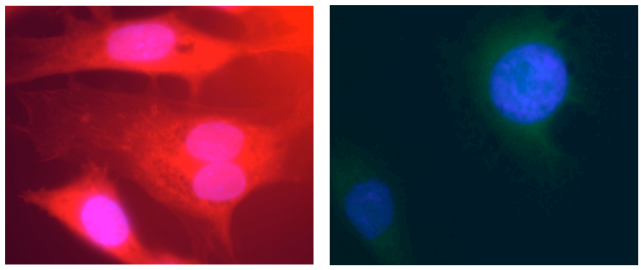


Figure 5- 3: NSLT cells replated from suspension show an increase in giant nuclei.

(A) Percentage of giant nuclei and binucleate cells in attached (att) and recently replated suspension (susp) cultures, stained with CellTracker™ and Hoechst. Giant nuclei were judged by eye, being at least twice the size of normal nuclei. Binucleate cells were identified using the cytoplasmic stain to define cells containing two nuclei, as shown below. Each sample count was from at least 100 cells on 2 coverslips. Graph shows the mean results from 3 experiments. Error bars indicate s.e.m. *** $p<0.02$, significant difference (2-tailed, unpaired t-test).

(B) Example micrographs of a binucleate cell (left) and a giant nucleus (right). DNA is stained with Hoechst (blue), and the cytoplasm is stained with CellTracker™ Red or Green.

potently and selectively inhibits CDK2, CDK1 and CDK5, by competing for the CDK's ATP-binding site (De Azevedo et al. 1997; Meijer et al. 1997). Initially, Roscovitine was tested on NS, NSLT and NSLTRas attached cells on coverslips at concentrations of 1, 2, 5, 10, 20, 50 and 100μM, and BrdU incorporation was quantified by immunofluorescence following a 4-hour BrdU pulse. 10μM was sufficient to inhibit BrdU incorporation in NS cells, while 50μM was required for a comparable inhibition in NSLT and NSLTRas cells, consistent with their higher levels of CDK2 and CDK1 activity (see Figure 4-2). However, to imitate the partial inhibition of DNA replication seen in NSLT suspended cells, a lower inhibitor concentration would be required. A second titration was then carried out using Roscovitine concentrations up to 45μM, and the effects on NSLT nuclei were analysed. Attached NSLT cells were treated every 24 hours with the inhibitor, and observed after 72 hours (Figure 5- 4A). Hoechst staining showed that a large proportion (15-22%) of NSLT cells had giant nuclei after 25-45μM Roscovitine treatment, suggesting overreplication similar to that occurring in NSLT suspended cells. The effects peaked at 35μM, higher concentrations having a lesser effect due to the inhibition of cell cycle progression. Figure 5- 4B shows an example of a 'giant' nucleus as seen in NSLT cells treated with 30μM Roscovitine.

The 35μM concentration of Roscovitine was then tested on cells analysed by flow cytometry. FACS profiles supported the idea of CDK inhibition inducing similar effects in attached cells as it does in suspension: profiles of both NS and NSLT attached cells treated with Roscovitine were similar to those of suspended cells (Figure 5- 4C). NS cells arrested predominantly in G1 phase, although Roscovitine treatment also arrested a few attached cells in G2, probably because CDK1 activity was insufficient to allow completion of mitosis. NSLT suspended cells instead had an aberrant 'proliferating' cell cycle profile, with a proportion of BrdU-incorporating cells and a significant minority which have an intermediate 'S-phase' DNA content but are BrdU-negative. The Roscovitine-treated attached NSLT cell profile was comparable to that of the untreated, suspended cells. Importantly, the dose of Roscovitine was crucial: while concentrations above 35μM effectively inhibited

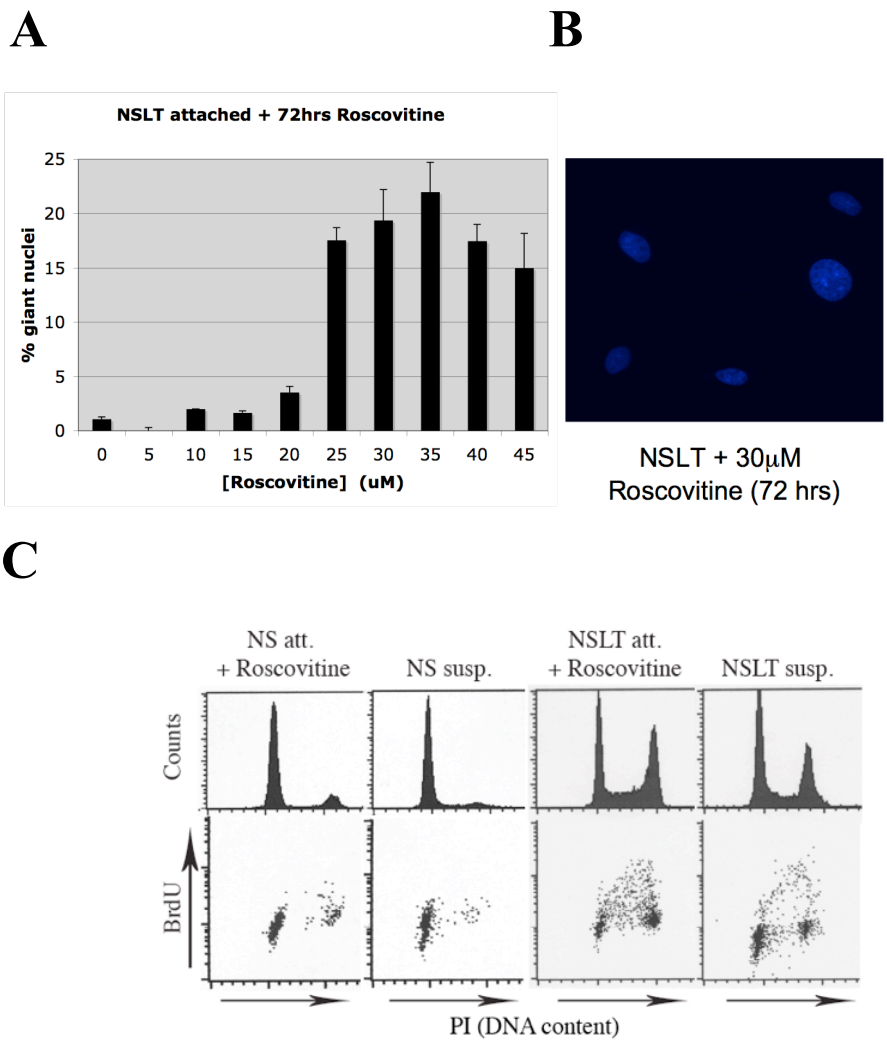


Figure 5- 4: CDK inhibition in NSLT attached cells produces a phenotype similar to that in suspended cells.

(A) Percentage of giant nuclei in NSLT cells treated for 72 hours with the indicated concentrations of the CDK1/CDK2 inhibitor Roscovitine, and stained with Hoechst. Inhibitor was dissolved in DMSO and added to medium. Medium containing inhibitor or DMSO control (0μM) was changed every 24 hours. Counts represent the mean of two treated coverslips, with at least 100 nuclei counted per coverslip.

(B) Fluorescence micrograph represents Hoechst-stained nuclei from NSLT cells treated with 30μM Roscovitine, showing one 'giant' nucleus, distinguishable by eye (centre right).

(C) FACS profiles of attached, Roscovitine-treated cells compared to untreated cells from 24-hour suspension culture for NS (left two panels) and NSLT (right two panels). Cells were stained with PI to indicate DNA content and BrdU incorporation measured to indicate DNA replication.

BrdU incorporation, lower concentrations had little effect on the cell cycle profile and all cells in S phase were able to actively incorporate BrdU during the one-hour pulse as in untreated cells. This raises the possibility that it is the partial inhibition of CDK activity that gives rise to the over-replication of DNA in suspended NSLT cells.

5.6 Genomic instability in NSLT suspended cells leads to oncogenic transformation

Variable chromosome number in a cell population is a sign of genomic instability. Genomic instability is considered to be a key factor in the progression of many cancers, acting to increase the frequency of oncogenic changes in cells (Loeb et al. 2008; Chandhok and Pellman 2009). A population of cells including both aneuploid and tetraploid chromosome complements would therefore be expected to exhibit a higher rate of oncogenic transformation than cells with a stable genome. We wanted to ask whether the genomic instability seen in our NSLT cells after prolonged periods in suspension was sufficient to induce full oncogenic transformation, including anchorage independence.

To test this, we took NSLT cells that had been in suspension for 72 hours, and cells that had been cultured in parallel under normal attached conditions, and put them into soft agar suspension to assay spontaneous colony formation. In preliminary experiments, neither cell type formed colonies after 3 weeks in soft agar, indicating that simply transferring cells from one form of suspension culture to another is not sufficient to induce oncogenic transformation. However, it would be predicted that some cell division would be required for genomic instability to produce the genetic changes required to drive oncogenic transformation. The simplest way to achieve this would be to replate cells following a period in suspension and allow them to resume proliferation with an abnormal genome for a time before assessing their transformation state. To test this, the soft agar experiment was repeated, using suspended cells cultured in attached conditions ("replated") for approximately 6 population doublings after retrieval from methylcellulose. NSLT attached cells passaged in parallel with those put into methylcellulose were used as a control.

Both “attached” and “replated” NSLT cells were put into soft agar culture. Strikingly, while the background from spontaneous mutation of “attached” NSLT cells remained negligible, the “replated” NSLT now formed colonies in suspension (Figure 5- 5A). Despite the colonies formed from the replated NSLT being rare, around 1 in 100,000 cells, there was a clear difference between these ‘destabilised’ cells and those passaged normally (Figure 5- 5B). An additional control population of NSLT cells was seeded into methylcellulose suspension and retrieved immediately, before passaging in parallel with the other cultures and seeding into soft agar. Colony formation in this population was as low as the attached cell control, indicating that it is the time in suspension, allowing aberrant genome replication, which is important for future oncogenic transformation and not the methylcellulose procedure itself.

To test the tumourigenic capacity of these unstable “replated” NSLT cells directly, they were injected subcutaneously into the flanks of nude mice. NSLT cells that had not been subject to a period in suspension, and NSLTRas cells, were used as negative and positive controls, respectively. While the positive control mice quickly developed subcutaneous tumours, and were sacrificed within two weeks, the NSLT-injected mice have not to date shown any signs of tumour development (5 months), and the experiment is ongoing.

5.7 Chapter summary and conclusions

In this chapter, we have investigated the development of genomic instability in NSLT cells following loss of anchorage. Quantification of >4N DNA content by flow cytometry indicated that DNA over-replication occurred in NSLT cells in the absence of anchorage, with up to 50% of cells having an above-diploid DNA content after 48 hours in suspension. Moreover, when replated and subjected to metaphase spread or Hoechst staining, at least 10% of these cells were aneuploid and a similar proportion had giant nuclei. Loss of anchorage was clearly responsible for these effects on the genome: >4N DNA content, giant nuclei and an aneuploid chromosome complement only appeared in suspended NSLT and not in attached cultures. Attached cells treated with the CDK inhibitor Roscovitine showed a similar overreplication phenotype, indicating that the reduced CDK activity in NSLT suspended cells is likely

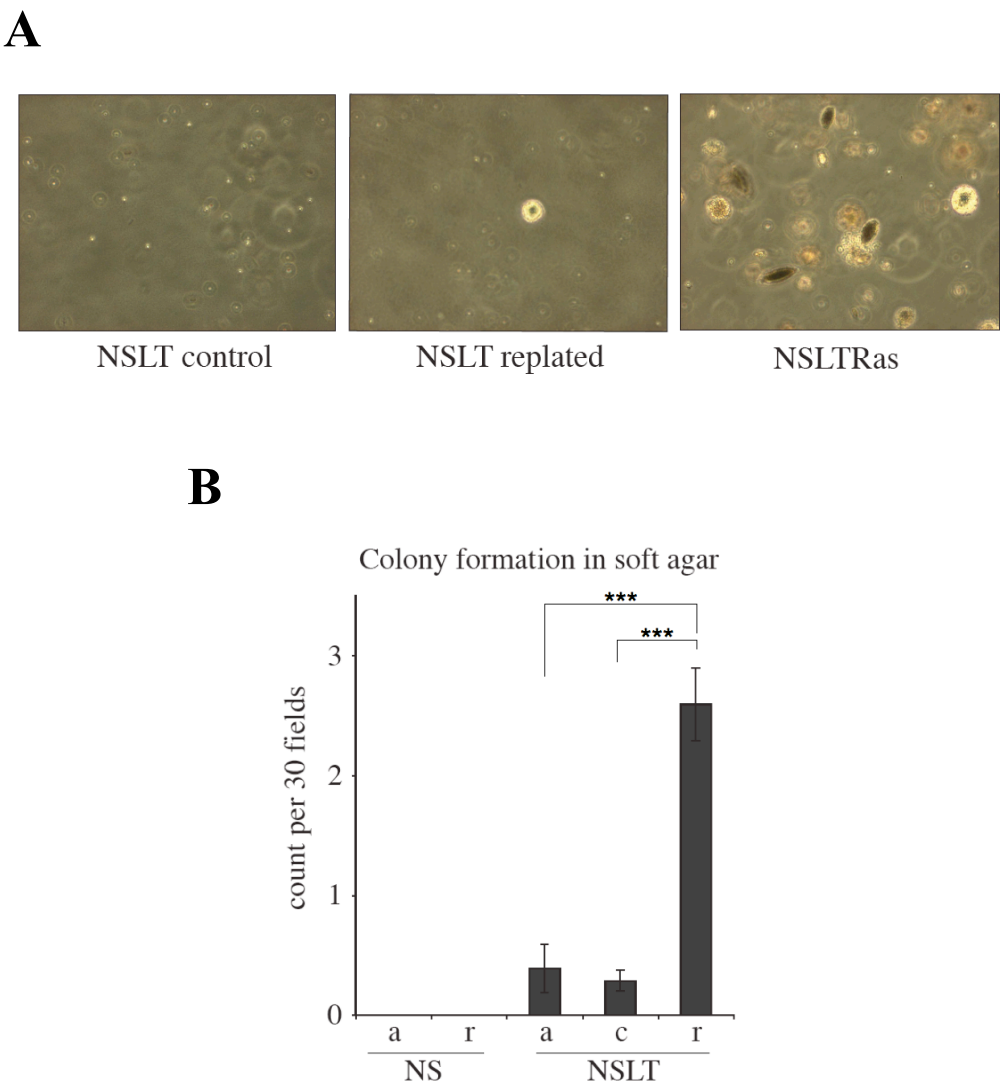


Figure 5- 5: NSLT cells passaged from suspension have an increased rate of oncogenic transformation

(A) Soft agar assay of colony formation: in NSLT cells after 72 hours in suspension, followed by replating (centre), compared with NSLT (left) and NSLTRas (right) passaged normally, as negative and positive controls respectively. (B) Quantification of combined results from six similar soft agar experiments. a = attached cells passaged normally; c = control cells passaged normally that have undergone the methylcellulose seeding and retrieval procedure; r = replated cells after 72-hour methylcellulose suspension, plus two additional passages on ordinary culture plates. Error bars indicate s.e.m. *** $p<0.0005$, significant difference (2-tailed, unpaired t-test).

responsible for development of the observed genomic abnormalities. These results indicate loss of anchorage in checkpoint-deficient cells as a novel contributor to the development of genomic instability.

We would expect a low rate of colony formation in genetically unstable cells from suspension, because, from the 10% of aneuploid NSLT cells detected after 48 hours, only a few may have survived the reattachment and been successfully expanded. They may have aborted cell cycle re-entry due to resumption of better genome controls in the presence of anchorage signals (Lewis et al. 2002; Truong et al. 2003), or the extra chromosomes may simply be too much of a burden for efficient proliferation (Torres et al. 2007; Weaver et al. 2007). In addition, of those that did resume proliferation with an unstable, aneuploid chromosome complement, not all changes would result in oncogene activation or loss of further cell cycle controls. The fact that colonies do form following expansion of genomically unstable cells, but not others, indicates an effective increase in the spontaneous mutation rate in unstable cell populations, thereby raising the likelihood of progression to a fully transformed and tumourigenic state. This result shows that a period without proper cell anchorage, which allows aberrant genome complements to develop, precipitates future transformation in checkpoint-deficient cells.

Ordinary NS cells, with functional p53 and Rb networks, are not susceptible to instability, since they arrest stably in G1 phase and do not undergo any DNA replication in suspension. NSLTRas cells, which can proliferate equally well in suspension as when attached, appear constitutively unstable, so loss of anchorage does not contribute to genomic instability in these cells. However, partly transformed cells such as NSLT, i.e. those lacking cell cycle checkpoints, are susceptible to increasing genomic instability in suspension. This shows that although the secondary, p27-mediated "anchorage checkpoint" induced in p53- and Rb-deficient cells prevents anchorage-independent proliferation, the fact that aberrant DNA replication is possible means that cells may develop an unstable genome more prone to oncogenic transformation. The checkpoint mechanism therefore acts as a "double-edged sword". Maintenance of anchorage dependence acts as a tumour suppressor in the short term, by inhibiting cell division, but the concomitant S phase

progression leads to the accumulation of genomic abnormalities, which can act to increase the rate of oncogenic transformation.

Chapter Six – Discussion

The maintenance and propagation of a stable genome through generations of cells is the major objective of the cell cycle process. To coordinate DNA replication with cell division, ensure proliferation is appropriate to the cellular context, and allow scope for repair to the genome following insult, mammalian cells have developed a range of cell cycle checkpoints which pause or arrest cell cycle progression in response to unfavourable conditions, such as insufficient mitogens or DNA damage (Elledge 1996). The failure of many of these checkpoint mechanisms has been shown to adversely affect genome stability and predispose to oncogenic transformation (Hartwell 1992; Bartek and Lukas 2001; Kastan and Bartek 2004; Kops et al. 2005). A key characteristic of transformed cells is their ability to proliferate anchorage independently. In this thesis I have analysed a primary cell model of transformation, to investigate how sequential loss of cell-cycle checkpoints results in anchorage independence. This model consists of three cell types:

- Normal Schwann cells (NS), which are anchorage dependent;
- NS cells expressing SV40 Large T antigen (NSLT), which inactivates p53 and Rb. Cells proliferate mitogen-independently, but remain anchorage dependent;
- NS cells expressing SV40 Large T plus oncogenic Ras (NSLTRas), which are fully transformed, and therefore proliferate anchorage-independently.

Using this model I have found that although NSLT cells fail to proliferate in suspension, they become genomically unstable after prolonged periods without anchorage. Importantly, this was not merely due to the abrogation of p53 and Rb checkpoints - loss of anchorage signals was necessary to initiate these genomic changes, which resulted in the subsequent emergence of fully-transformed cells. This indicates loss of anchorage as a novel trigger for the development of genome instability and transformation, in the absence of primary checkpoint mechanisms. These findings, and their implications, are discussed in more detail below.

6.1 Manifestations of the anchorage checkpoint in our system and role of p27

It is well established that adherent cells require both mitogens and signals from the extracellular matrix to proliferate. Epithelial and endothelial cells additionally require adhesion for survival, and these cell types respond to loss of anchorage signals by inducing a form of apoptosis, known as anoikis. Non-epithelial cell types, such as fibroblasts, do not die, but undergo a cell cycle arrest, following loss of anchorage (Otsuka and Moskowitz 1975; Frisch and Francis 1994). Previous studies on anchorage dependence in fibroblasts have shown that attachment signals are required to induce cyclin D1, promote the expression of cyclins E and A, and the activation of CDK2, to allow cell cycle progression past the Rb/E2F checkpoint (Guadagno and Assoian 1991; Guadagno et al. 1993; Fang et al. 1996; Zhu et al. 1996). Detached cells therefore fail to enter S phase and undergo a G1 arrest, due to insufficient cyclin D1 induction. Moreover, in the absence of anchorage, the CDK inhibitor p27 is stabilised, contributing to the maintenance of the arrest by direct inhibition of cyclin E-CDK2 activity and suppression of cyclin A transcription (Schulze et al. 1996; Zhu et al. 1996).

Although Large T antigen can transform some immortalised cell lines, expression of LT is rarely sufficient to induce anchorage-independent proliferation in primary cells. Full transformation of human cells has been shown to require SV40 small t antigen in addition to LT, hTERT and oncogenic Ras (Hahn et al. 2002). Small t was found to be necessary for p27 downregulation (Porrás et al. 1999; Schüchner and Wintersberger 1999). More recently, small t was reported to cooperate with cyclin E overexpression to overcome p27-induced arrest, and to activate integrin signalling in human cells, leading to anchorage-independent proliferation in cooperation with LT (Moreno et al. 2004; Sotillo et al. 2008). In rodent cells, which constitutively express telomerase, oncogenic Ras has been shown to cooperate with p53 and Rb loss to induce anchorage independence (Hicks et al. 1991; Serrano et al. 1997; Mitchell et al. 2003). Previous reports have shown that Ras exerts its effect in part by promoting p27 degradation (Aktas et al. 1997; Kawada et al. 1997), as well as

activating the ERK pathway needed for cyclin D1 induction. These reports suggest the existence of a secondary, Rb-independent anchorage checkpoint mediated by p27 in LT-expressing cells.

Consistent with previous findings in fibroblasts, NS cells in suspension did not undergo anoikis, but arrested with a G1 DNA content. NS cells were able to return to the cell cycle upon replating, indicating that the arrest was reversible. G1 arrest in suspension was characterised by loss of cyclin D1, cyclin E, and cyclin A expression, and minimal cyclin-dependent kinase activity. Moreover, p27 was also strongly upregulated in suspension, in agreement with results in other cell types.

As in other rodent cells, Ras cooperated with the inactivation of p53 and Rb by LT to promote proliferation of suspended NSLTRas cells. Importantly, these cells failed to induce p27 in suspension, consistent with previous results suggesting that Ras-induced p27 proteolysis is responsible for anchorage-independent proliferation (Kawada et al. 1997). Moreover, data from our laboratory indicates that an active Raf kinase domain is sufficient to induce anchorage-independent proliferation in LT-expressing cells, suggesting that Ras is acting through Raf to overcome the anchorage checkpoint (D. Danovi, unpublished). However, we cannot rule out other Ras-induced mechanisms that might also be involved in promoting anchorage-independent proliferation, and these are discussed in section 6.3.

As anchorage dependent cells, Schwann cells expressing LT alone failed to proliferate in suspension, and accordingly, cyclin-CDK activity was strongly inhibited. This was found to be due to the upregulation of p27, as there was an increase in p27 bound to cyclin-CDK complexes in NSLT suspended cells. In addition, inactivation of p27 in LT-expressing MEFs induced colony formation in soft agar, demonstrating directly that p27 mediates a secondary anchorage checkpoint in cells where Rb is inactivated. These results reaffirm p27's importance as a tumour suppressor. Many studies have demonstrated that downregulation, mislocalisation, and increased degradation of p27 via Skp2 are correlated with poor prognosis in a range of cancers (Catzavelos et al. 1997; Loda et al. 1997; Chu et al. 2008). Notably, a mouse model of prostate cancer

has shown that p27 deficiency accelerates progression to a poorly differentiated carcinoma (PDCA) in animals expressing SV40 LT in the prostate gland (Shaffer et al. 2005). This report is particularly interesting in the light of our results, since progression to PDCA is associated with loss of E-cadherin, and therefore a change in the adhesive microenvironment of the cell (Umbas et al. 1992; Perl et al. 1998). It is tempting to speculate that the prostate cells expressing LT in the absence of p27 would be anchorage independent, thus encouraging tumourigenesis.

In contrast with NS and NSLTRas cells, NSLT cells also exhibited new and unexpected behaviour in suspension. Although proliferation in these cells was inhibited, DNA replication was not completely blocked. This indicated that in the absence of the Rb checkpoint, LT cells were able to overcome G1 arrest, consistent with previous observations (Mann and Jones 1996). Moreover, overreplication occurred if suspension was prolonged more than a few hours, suggesting that p27 is not sufficient to fully arrest or control the cell cycle in the absence of the Rb checkpoint. NSLT suspended cells also exhibited aneuploidy, and an increase in the rate of spontaneous transformation. These findings suggest that in checkpoint-deficient cells, upregulation of p27 may indirectly act to promote tumourigenesis by causing genome instability.

A few studies have already linked p27 to genomic instability, but reports have been conflicting. For example, one study found that p27 accumulation resulted in overreplication, in conjunction with overexpression of cyclin E and A, which is consistent with our results (Nakayama et al. 2000). However, others suggest that overexpression of p27 inhibits the emergence of aneuploidy in cancer cells (Chen et al. 1996b), or that p27 deficiency leads to an increase in genomic instability (Payne et al. 2008). How can this discrepancy be resolved? One possibility is that a balanced oscillation of CDK activity, rather than overall activation or inhibition, is crucial for correct control of DNA replication, and the effect of increasing p27 therefore depends on cellular context. Our results additionally suggest that checkpoint status is key in determining the outcome of increased p27: in conjunction with the Rb checkpoint, p27 strengthens G1 arrest and therefore safeguards genome stability; in the absence of Rb function, p27

may be counterproductive, due to the adverse effects of inhibiting CDK activity during S phase, as discussed below.

6.2 How p27 might induce genome instability

Our results suggest that aberrant DNA replication in NSLT cells is caused by p27 upregulation in suspension inhibiting CDK activity, in the absence of Rb and p53 checkpoints. This is supported by the analogous results obtained using the CDK inhibitor Roscovitine in attached NSLT cells. How could CDK inhibition give rise to genomic instability? Two potential mechanisms are origin re-licensing, and replication stress, as described below.

6.2.1 Origin re-licensing

Replication licensing is normally strictly confined to late mitosis and early G1 phase, to ensure that each section of DNA is replicated only once (Nishitani and Lygerou 2002). Moreover, there is evidence that deregulation of replication licensing proteins contributes to aneuploidy and instability of chromosome structure in cancer cells and mouse models (Hook et al. 2007; Blow and Gillespie 2008). CDK activity plays an important role in controlling origin licensing in mammalian cells, as well as in yeast (Itzhaki et al. 1997; Bates et al. 1998; Coverley et al. 1998; Nguyen et al. 2001; Ballabeni et al. 2004; Liu et al. 2004a; Porter 2008). Robust switching between states of high and low CDK activity minimises the time where activity is 'intermediate' and the cell is vulnerable to inappropriate origin licensing or firing (Diffley 2004). Completion of mitosis, with its associated drop in CDK activity, is normally required to 'reset' pre-replicative complexes at replication origins (Stillman 1996), suggesting that an unscheduled reduction in CDK activity may permit origin re-licensing before sister chromatids are separated at mitosis, resulting in overreplication. Thus, in NSLT suspended cells, partial inhibition of CDK activity during S and G2 phases could cause inappropriate origin licensing and re-firing, leading to an increased, unstable DNA content. An alternative and non-mutually exclusive possibility is that anchorage is itself required for regulation of replication licensing components: for example, cdc6 has been shown to require anchorage for its expression in fibroblasts (Jinno et al. 2002).

This could impair licensing control in detached cells such that origin firing is uncoordinated, leading to incomplete replication, again producing an unstable genome.

6.2.2 Replication stress

The second potential link between loss of anchorage and oncogenic transformation could be replication stress, and consequent activation of a chronic DNA damage response, in NSLT suspended cells. Replication stress has been reported in cancer cells as a result of the conflict between oncogene stimulation of proliferative pathways, and normal cell cycle checkpoints (Bartkova et al. 2006; Di Micco et al. 2006). Importantly, this stress has been shown to occur very early in the tumourigenic process, and it is associated with DNA damage, as shown by characteristic markers such as γ-H2AX and activated Chk2 (Bartkova et al. 2005; Gorgoulis et al. 2005). Loss of anchorage in NSLT cells could result in replication stress due to a similar conflict between loss of Rb activity, promoting proliferation, and p27-induced CDK inhibition, acting to impede cell cycle progression. CDK inhibition in S phase would be predicted to result in the stalling of replication forks, and activation of a DNA damage response due to collapsed forks and unreplicated DNA. The irregular BrdU incorporation in suspended NSLT cells suggests that replication forks may indeed be stalling in S phase in response to CDK inhibition.

Several observations suggest that LT-expressing cells with inactive p53 and Rb may be prone to genomic instability following replication stress. Firstly, the absence of functional p53 removes the p21 checkpoint in G2 phase, allowing progression into mitosis in the presence of DNA lesions. Rb loss has been shown to impair DNA damage-induced arrest and promote DNA double-strand break formation (Sage et al. 2000; Pickering and Kowalik 2006). Cells lacking Rb activity will overreplicate in response to cell cycle inhibition by either p21 or nocodazole, suggesting that p27 could have the same effect in NSLT cells (Niculescu et al. 1998; Srinivasan et al. 2007). p53 has been shown to prevent rereplication in response to cdt1 and cdc6 overexpression (Vaziri et al. 2003), and both p53 and Rb have been shown to be required for the

'tetraploidy checkpoint' arrest following mitotic slippage (Iida et al. 2004). Finally, SV40 LT alone has recently been shown to promote a DNA damage response (Hein et al. 2009). Although we have not directly assessed DNA damage in our NSLT cells, the chromosome complement was stable and overreplication did not occur in attached cells: loss of anchorage was required to induce these changes. This suggests either that LT is not sufficient to induce DNA damage in our system, or more interestingly that any damage incurred in attached cells is repairable, but detachment of the cell from the substratum and consequent CDK inhibition triggers replication stress and chronic damage. In support of this idea, adhesion is required for arrest or apoptosis following DNA damage in fibroblasts (Gadbois et al. 1997; Lewis et al. 2002; Truong et al. 2003). This suggests that loss of anchorage could impair the damage response, as well as causing replicative stress. An impaired DNA damage response in the presence of CDK inhibition has already been demonstrated in human cells, using the inhibitor drug Roscovitine (Jazayeri et al. 2005).

6.3 Alternative mechanisms of Ras-induced anchorage independence

The finding that p27 is responsible for preventing anchorage-independent proliferation in LT-expressing cells is neatly balanced by previous observations showing that oncogenic Ras signalling downregulates p27 via Skp2-mediated degradation, as shown by the lower p27 levels in NSLTRas cells (Kawada et al. 1997). This provides a ready explanation for the mechanism by which Ras evades the anchorage checkpoint in this system. However, it is unlikely that this is the whole story. Our finding that CDK activity is somewhat inhibited in NSLTRas suspended compared to attached cells, despite no upregulation of p27, points to the existence of an alternative anchorage-dependent mechanism of regulating CDK activity. Moreover, the colony-forming ability of cells expressing LT and oncogenic Ras is higher than that of cells expressing LT in the absence of p27 inhibition. These observations suggest that Ras promotes anchorage-independent proliferation in other ways, in addition to downregulation of p27. Two other effects of Ras that are likely to be relevant to anchorage independence are described below.

6.3.1 p73 isoform switching

The p53-related transcription factor p73 is found in two forms: an oncogenic "ΔN" form which cooperates with Ras to transform cells in the same way as loss of p53, and the tumour-suppressive "TA" or transactivation-competent form (Grob et al. 2001; Petrenko et al. 2003). Importantly, although p53 and p73 are related, LT does not bind or inhibit p73 (Marin et al. 1998). Beitzinger et al., using a model system that, like ours, employs LT and oncogenic Ras, found that the Ras-mediated switch between anchorage-dependent and independent proliferation was associated with a switch from expression of the TA form to the ΔN form of p73, via PI3K signalling (Beitzinger et al. 2008). This study further demonstrated that knockdown of the TAp73 isoform was sufficient to induce anchorage-independent proliferation in the LT-expressing cells, and either re-expression of TAp73, or depletion of ΔNp73 prevented anchorage-independent proliferation in the Ras-expressing cells. Since the obvious difference between the two p73 isoforms is their transcriptional activity, Beitzinger et al. also attempted to define relevant p73 target genes that would explain the maintenance of anchorage dependence in their cells, though none so far has yielded a plausible explanation. Given the precedent set by p53 and its control of the CDK inhibitor p21, the possibility remains that an important functional target of p73 involved in anchorage dependence will be found.

Related to this, and highly relevant to our results, is the recent discovery that TAp73 also plays a role in maintaining genomic stability, in particular suppressing aneuploidy, especially when p53 is absent (Talos et al. 2007; Tomasini et al. 2008). Furthermore, previous work has indicated that adhesion is required for the DNA-damage-induced stabilisation of p73 (Lewis et al. 2002; Truong et al. 2003). These combined results have intriguing parallels with our findings, and suggest that p73 destabilisation may contribute to the genomic instability of NSLT cells in suspension.

6.3.2 Role of the cytoskeleton in successful cell division and survival

A recent report suggests that normal human fibroblasts can complete mitosis but not cytokinesis in suspension, leading to binucleation of suspended cells, and that Ras acts to promote cytokinesis, rescuing this phenotype (Thullberg et al. 2007). Although this did not seem to be the case in our system, since we did not observe a large increase in binucleate cells, the idea that cytokinesis may be a problem for suspended cells is intriguing, and suggests a particular role for altered cytoskeletal dynamics in Ras-transformed cells. Since oncogenic Ras counts many cytoskeletal regulators amongst its effectors, including Rho and Rac (Marshall 1996; Rodriguez-Viciana et al. 1997; Klein et al. 2008), it is not unreasonable to predict that altered cytoskeletal dynamics contributes to the anchorage-independent proliferation of Ras-expressing cells by supporting cell division in suspension.

The differing cytoskeletal conformations in NSLT and NSLTRas cells are suggested by their different morphologies in attached culture (see Figure 3-1). Cell shape, as well as integrin ligation, has been shown to be important for cell-cycle progression in anchorage-dependent cells (Huang et al. 1998; Mammoto et al. 2004). Cytoskeletal changes are also well known to affect cell division. For example, a stiffened actin cortex during cell rounding is required for correct formation and orientation of the spindle at mitosis (Kunda et al. 2008). Both attached and suspended cells are rounded when entering mitosis, but attached cells remain tethered at key opposing points by narrow cytoplasmic projections (Mitchison 1992). The correct positioning and stability of the mitotic spindle is dependent upon balanced forces being set up between these projections, and if the cell shape is artificially distorted, cells may develop multipolar spindles and suffer unequal chromosome segregation (Grill and Hyman 2005; Théry et al. 2007). In suspension cultures such as methylcellulose and soft agar, the cell has lost these orientated connections, and therefore the spindle positioning may be aberrant, leading to aneuploidy. Furthermore, generation of the forces involved in pulling chromosomes to opposite spindle poles may require altered cytoskeletal organisation and tension in the absence of such substrate tethering. This could result in mitotic arrest in

cells lacking the required cytoskeletal dynamics for division in suspension. If attempts to complete mitosis in suspension were not successful after a certain amount of time, similarly to the response to a delay induced by spindle poisons, the cell could either die, or undergo a mitotic 'slippage', returning to G1 with a tetraploid set of chromosomes (Verdoodt et al. 1999; Chen et al. 2003b). This is an attractive explanation for both the increase in cell death seen in NSLT cells after prolonged periods in suspension, and the emergence of tetraploid and aneuploid cells. We can also speculate that the lower the rigidity of the surrounding matrix, the lower the tension in the cytoskeleton and the more difficult it would be for a cell to complete mitosis. This idea is supported by the increased survival of cells observed in soft agar, a more solid support compared to methylcellulose suspension.

6.4 In vivo relevance and implications for cancer therapy

To our knowledge, this is the first report demonstrating that loss of normal anchorage signals can lead to genomic instability and promote cellular transformation. Based on these results, it is conceivable that the detachment of a checkpoint-deficient but non-cancerous cell from its normal environment (or simply a change in cell adhesion) could precipitate tumourigenesis, due to the development of genomic instability. Should the cell detach from the tumour mass completely, this effect could promote tumourigenesis in a new location. This concept is supported by the finding that metastasis of single cells is not always a late event in tumour progression, and can occur before the cell has fully transformed (Hüsemann et al. 2008).

In the present case, genome instability was triggered in checkpoint-deficient cells by the induction of p27 following loss of normal anchorage signals. However, it would be predicted that inhibiting CDK activity by other mechanisms would have a similar effect. Consistent with this, we found that the addition of moderate doses of the CDK inhibitor Roscovitine to checkpoint deficient cells when attached, produced an impaired DNA replication phenotype similar to that induced in our suspended cells. These inhibitors have been developed in part as potential cancer therapeutics, several of which are currently in clinical trials, including Roscovitine itself (Malumbres et al. 2008;

Malumbres and Barbacid 2009). Additionally, a novel p27-stabilising compound, argyrin A, has recently been identified as a potential cancer therapeutic, which prevents p27 degradation by inhibition of the proteasome and so would also be predicted to inhibit CDK activity (Nickeleit et al. 2008). Our results suggest that doses of CDK inhibitors insufficient to completely arrest cell-cycle progression may have deleterious consequences, particularly in checkpoint-deficient cells, by promoting gross chromosomal instability, which can increase the chances of tumour progression. This could have implications for the long-term outcome of drug treatment, especially in patients with primary tumours that lack key tumour suppressor genes.

6.5 Further work

The broad interpretation of the results presented in this thesis indicates a novel link between disruption of cell adhesion and increased transformation. Further work should aim to strengthen the mechanistic links between loss of anchorage signals, genome instability and tumourigenesis. For example, extrapolating the results showing soft agar colony formation following temporary disruption of anchorage, to show that a period in suspension is sufficient to cause NSLT cells to form tumours in nude mice, would more firmly establish this connection.

More molecular detail for the mechanism linking CDK inhibition and aberrant DNA replication is also needed. Analysis of proteins involved in the formation of the pre-replication complex, such as MCMs, Cdt1 and geminin, would help identify any abnormalities in DNA licensing, and also determine whether the expression of any licensing components (other than cdc6) is anchorage dependent. Additionally, detection of markers of DNA damage, such as γ-H2AX or phosphorylated Chk2, would show whether the overreplication seen in LT-expressing cells in suspension triggers a DNA damage response consistent with replication stress, and whether any damage incurred is repairable on reattachment of the cells.

The induction of DNA overreplication and aneuploidy upon suspension of LT-expressing cells should be demonstrated in other cell types, as although features of the Schwann cell model have been reproduced in other systems, loss

of anchorage may not represent a general mechanism of producing genomic instability in all cells. For example, it would be interesting to see whether an 'immortalised' epithelial cell type would exhibit a similar incomplete arrest and overreplication in suspension. As well as being a more accurate model for the majority of cancers, which usually arise in epithelial tissues, this would also enable studies correlating the loss of E-cadherin and p27 downregulation with anchorage-independent proliferation, as has been done for contact inhibition (Motti et al. 2005).

Our results implicating CDK inhibition in the development of genome instability used Roscovitine, which inhibits both CDK1 and CDK2 activity. An inhibitor more specific to CDK2 could be used to clarify the role of particular CDKs in rereplication and aneuploidy. Investigation of other cell cycle kinases important in mitosis and the spindle checkpoint, for example Aurora, could further elucidate how aneuploid NSLT cells arose in suspension. A more detailed time course assay of kinase activity in NS cells directly after seeding in suspension would show whether CDK activity is maintained following loss of anchorage, to allow completion of mitosis and return to G1.

Finally, other mechanisms that have been shown to induce anchorage independence in LT-expressing cells, such as expression of the ΔNp73 isoform, could be analysed in our system in comparison with oncogenic Ras expression. This would help isolate genetic targets representing the minimum requirements for anchorage-independent proliferation in mammalian cells.

In summary, the results detailed in this study have highlighted a wider role for anchorage in cell cycle control. Further work should establish the effects of altered cell attachment in different systems, and reveal the full implications for genomic stability and cancer.

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